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Evidence for BCR-ABL oncoprotein packaging into extracellular vesicles released by BCR-ABL$^+$ leukemia cells: possible implications on cellular response to STI571

Alexandra Isabel Lopes Teixeira

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Dissertação de Candidatura ao grau de Mestre em Oncologia – Especialização em Oncologia Molecular, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Prof. Doutora Maria Helena da Silva de Vasconcelos Meehan

Categoria – Professor Auxiliar

Afiliação – Departamento de Ciências Biológicas da Faculdade de Farmácia da Universidade do Porto e Coordenadora do Grupo Cancer Drug Resistance do Instituto de Patologia e Imunologia Molecular da Universidade do Porto/Instituto de Investigação e Inovação em Saúde da Universidade do Porto

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Afiliação – Grupo Cancer Drug Resistance do Instituto de Patologia e Imunologia Molecular da Universidade do Porto/Instituto de Investigação e Inovação em Saúde da Universidade do Porto

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The noblest pleasure is the joy of understanding.

Leonardo da Vinci

Scientific presentations in the framework of this work

- **Abstract published in book of abstracts**

- "Is there intercellular transfer of drug resistance mediated by Extracellular Vesicles in a Chronic Myeloid Leukemia cell line with mutated *BCR-ABL*?" Teixeira A., Sousa D., Xavier C. P. R., Vasconcelos M. H. Poster presented at the10th IJUP – Encontro de Investigação Jovem da Universidade do Porto, February 2017, Porto, Portugal

- **Scientific presentation without publication of abstract**

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Index

Index of Figures

Index of Schemes

Index of Tables

Index of Annexes

Abbreviations

A

ABC	ATP-binding cassette
ABL1	ABL proto-oncogene 1, non-receptor tyrosine kinase
ATP	Adenosine triphosphate

B

BCR	BCR, RhoGEF and GTPase activating protein
BRAF	Serine/threonine-protein kinase B-raf
BSA	Bovine serum albumin

C

CAF	Cancer associated fibroblast
CCyR	Complete cytogenetic response
CD	Cluster of differentiation
cDNA	Complementary DNA
CDR	Cancer drug resistance
CHMP4B	Charged Multivesicular Body Protein 4B
CHR	Complete hematologic response
CML	Chronic myeloid leukemia
CMR	Complete molecular response
cyt *c*	Cytochrome *c*

D

DLS	Dynamic Light Scattering
DMSO	Dimethyl sulfoxide

E

EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediamine-tretaacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular vesicle

F

FBS	Fetal bovine serum
FDA	Food and Drug Administration
FISH	Fluorescence in situ hybridization

G

G-CSF	Granulocyte-colony stimulating factor

H

hOCT1	Human organic cation transporter 1
HSC	Hematopoietic stem cell
HSP	Heat-shock protein

I

IAP	Inhibitor of apoptosis protein

IFN-α	Interferon-α
Ile	Isoleucine

J

JNK	c-jun N-terminal kinase

K

KIT	KIT protein
KRAS	KRAS protein

L

lncRNA	Long noncoding RNA
LSC	Leukemia stem cell

M

MAPK	Mitogen activated protein kinase
MDR	Multidrug resistance
MHC	Major histocompatibility complex
miRNA	microRNA
MPP	Multipotent progenitor
mRNA	Messenger RNA
MRP-1	Multidrug resistance-associated protein-1
MSC	Mesenchymal stem cell
MVB	Multivesicular body

N

NaCl	Sodium chloride
NF-kB	Nuclear factor kappa B
NTA	Nanoparticle Tracking Analysis

P

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF-R	Platelet-derived growth factor receptor
Ph	Philadelphia
PI-3	Phosphoinositide-3
P-loop	Phosphate- or ATP-binding loop
PMSF	Phenylmethylsulfonyl fluoride

Q

qRT-PCR	Quantitative real time-polymerase chain reaction

R

Rab	Ras-associated binding protein
RT	Room temperature

S

SAPK	Stress-activated protein kinase
SH2	Src-homology-2
SH3	Src-homology-3
siRNA	Small interfering RNA
SNARE	Soluble *N*-ethylmaleimide-sensitive factor activating protein receptor
STAT	Signal transducers and activators of transcription

T

TAM	Tumor-associated macrophage
TBE	Tris/Borate/EDTA
TC	Tumor cell
TEM	Transmission Electron Microscopy
Thr	Threonine
TKI	Tyrosine kinase inhibitors

W

WB	Western Blot

Abstract

Chronic Myeloid Leukemia (CML) is a hematological malignancy, characterized by the Philadelphia chromosome, resulting from the t(9;22)(q34;q11.2) translocation, which generates the *BCR-ABL* fusion gene. This genetic alteration originates a constitutively active tyrosine kinase, deregulating multiple downstream pathways and, thus, promoting cellular survival and proliferation. The gold standard treatment of BCR-ABL$^+$ CML is the use of BCR-ABL inhibitors. Imatinib mesylate (STI571, Gleevec) is one of the most widely used drugs as first line treatment in the clinical practice. Unfortunately, even though these drugs are very effective for disease management, resistance to these molecules often arises (mostly due to mutations on the catalytic BCR-ABL domain, which is the target of these drugs), representing a major clinical drawback in the treatment of these patients. Recent studies have indicated that drug resistance may be horizontally transferred by extracellular vesicles (EVs), such as microvesicles and exosomes, from donor to recipient cells. Interestingly, is it known that EVs released by donor drug-resistant cells contain information that reflects the cells of origin, such as proteins, mRNAs or microRNAs.

The main objectives of this work were to ascertain if: i) EVs released by BCR-ABL$^+$ leukemia cell lines had BCR-ABL (protein and mRNA) on their cargo; and ii) EVs shed by a drug-resistant BCR-ABL$^+$ cell line (KBM5-STI, with mutant *BCR-ABL* in T315I, which confers resistance to STI571) were responsible for intercellular transfer of a drug-resistance phenotype to their drug-sensitive counterpart cells (KBM5).

To address this, the dose-response curves to STI571 of the above-mentioned pair of counterpart drug resistant and drug sensitive *BCR-ABL*$^+$ cell lines were first performed, using the resazurin assay. EVs were then isolated from those cells using the ultracentrifugation protocol and were characterized by Dynamic Light Scattering (DLS), Nanoparticle Tracking Analysis (NTA), Transmission Electron Microscopy (TEM) and Western Blot (WB). The cargo of the EVs regarding BCR-ABL content was assessed by WB (and also preliminary assessed by quantitative Real Time-Polymerase Chain Reaction – qRT-PCR – which was performed by collaborators of the research team). Finally, the response of drug-sensitive cells to STI571 following co-culture with EVs shed by drug-resistant cells was verified with the resazurin assay.

The distinct growth of the two cell lines studied regarding their response to STI571 was confirmed, being the IC50 of the drug resistant cell line about 100 times higher than the IC50 of the sensitive one. EVs characterization proved their successful isolation: the size distribution was the expected for these particles (10 – 1000 nm); their morphology was

observed by TEM; and they presented several classical markers, with no evidence of cellular contaminants. Interestingly, BCR-ABL oncoprotein was detected in EVs released by both cell lines, with a stronger detection signal (on Western Blots) than the one found in the cells, for the same amount of protein analyzed. Thus, these results suggest that there might be a selective packaging of BCR-ABL into EVs, promoting a mechanism for oncoprotein shedding. Preliminary results obtained in collaboration with other researchers also showed the presence of BCR-ABL mRNA in those EVs. Nevertheless, under the conditions tested, the EVs released by KBM5-STI (resistant) cells did not influence the response of drug-sensitive recipient cells to STI571. In summary, under the conditions tested, the work presented in this thesis found no evidence for a transfer of drug resistance, mediated by EVs, from resistant to sensitive cells.

Keywords:

Chronic Myeloid Leukemia | BCR-ABL | Imatinib mesylate or STI571 | Drug resistance | Extracellular Vesicles | Horizontal transfer of drug resistance

Resumo

A leucemia mieloide crónica (LMC) é uma doença maligna hematológica, caracterizada pelo cromossoma de Filadélfia, resultante da translocação t(9; 22)(q34; q11.2), que origina o gene de fusão *BCR-ABL*. Esta alteração genética dá origem a uma tirosina cinase constitutivamente ativa, desregulando múltiplas vias a jusante, promovendo assim a sobrevivência e proliferação celulares. O tratamento padrão da LMC BCR-ABL$^+$ baseia-se no uso de inibidores de BCR-ABL. O *imatinib mesylate* (STI571, Gleevec) é um dos fármacos mais amplamente utilizados como tratamento de primeira linha na prática clínica. Infelizmente, apesar da eficácia destes fármacos no controlo da doença, surgem frequentemente casos de resistência a estas moléculas (principalmente devido a mutações no domínio catalítico do BCR-ABL, sendo esse o alvo destes fármacos), representando a maior desvantagem clínica no tratamento destes pacientes. Estudos recentes indicaram que a resistência a fármacos pode ser transferida horizontalmente por vesículas extracelulares (EVs), tais como microvesículas e exossomas, de células dadoras para células recetoras. Curiosamente, sabe-se que as EVs libertadas por células dadoras resistentes a fármacos contêm informação que reflete as células de origem, como proteínas, mRNAs ou microRNAs.

Os principais objetivos deste trabalho foram verificar se: i) as EVs libertadas por linhas celulares leucémicas BCR-ABL$^+$ continham BCR-ABL (proteína e mRNA) no seu interior; e ii) as EVs libertadas por uma linha celular BCR-ABL$^+$ resistente ao fármaco (KBM5-STI, com BCR-ABL contendo a mutação T315I, que confere resistência ao STI571) seriam responsáveis pela transferência intercelular do fenótipo de resistência ao fármaco, para as correspondentes células sensíveis (KBM5).

Para tal, curvas dose-resposta ao STI571 do supra-mencionado par de linhas celulares BCR-ABL$^+$, sensível e resistente ao fármaco, foram inicialmente determinadas usando o ensaio da resazurina. De seguida, as EVs foram isoladas destas células usando o protocolo de ultracentrifugação e foram caracterizadas por diferentes métodos: *Dynamic Light Scattering* (DLS), *Nanoparticle Tracking Analysis* (NTA), Microscopia Eletrónica de Transmissão (TEM) e *Western Blot* (WB). O conteúdo das EVs relativamente à presença de BCR-ABL foi avaliado por WB (e também avaliado preliminarmente por *Real Time-Polymerase Chain Reaction* quantitativo – qRT-PCR – realizado por colaboradores do grupo de investigação). Finalmente, a resposta das células sensíveis ao STI571 após co-cultura com EVs libertadas pelas células resistentes ao fármaco foi verificada, usando o ensaio da resazurina.

O crescimento distinto das duas linhas celulares estudadas relativamente à sua resposta ao STI571 foi confirmado, sendo o IC50 da linha celular resistente cerca de 100 vezes maior do que o IC50 da sensível. A caracterização das EVs provou o sucesso do seu isolamento: a distribuição de tamanhos foi a esperada para estas partículas (10 - 1000 nm); a sua morfologia foi observada por TEM; e apresentaram vários marcadores clássicos de EVs, sem evidências de contaminantes celulares. Curiosamente, a oncoproteína BCR-ABL foi detetada nas EVs libertadas por ambas as linhas celulares, com um sinal de deteção mais forte (nos Western Blots) quando comparado com o encontrado nas células, para a mesma quantidade de proteína analisada. Assim, estes resultados sugerem que poderá haver um empacotamento seletivo de BCR-ABL nas EVs, promovendo um mecanismo de libertação da oncoproteína. Os resultados preliminares obtidos em colaboração com outros investigadores também demonstraram a presença de mRNA de BCR-ABL nas EVs. No entanto, nas condições testadas, as EVs libertadas pelas células KBM5-STI (resistentes) não influenciaram a resposta ao STI571 das células recetoras sensíveis ao fármaco. Em suma, nas condições testadas, o trabalho apresentado nesta tese não encontrou evidências de transferência de resistência a fármacos, mediada por EVs, de células resistentes para células sensíveis.

Palavras-chave:

Leucemia Mieloide Crónica | BCR-ABL | Imatinib mesylate ou STI571 | Resistência a fármacos | Vesículas Extracelulares | Transferência horizontal de resistência a fármacos

I. Introduction

1.1. Cancer – a general overview

In the last decades, cancer has become an emergent and serious public health problem worldwide [1]. According to data from the World Health Organization, an increase to 17.1 million new cases of cancer is predicted in 2020 (against 14.1 million cases in 2012). The numbers regarding mortality are also worrisome: 10.0 million deaths due to cancer are estimated for 2020 [2, 3]. Therefore, research on cancer is of great importance in order to reduce such devastating impact.

Cancer is a heterogenous and multifactorial disease, about which much has been studied; however, despite growing research in the area and promising advances, much remains to be unveiled [4]. The rise in the incidence of cancer, in developed countries, is mostly due to ageing and lifestyle habits (such as smoking, consumption of alcohol, obesity and sedentarism), which represent risk factors for this disease. The substantial increase in the number of cancer cases in recent years is also related with the improvement of diagnostic methods. However, an early detection of this disease has an important impact on treatment success and survival of patients [4, 5].

The interaction between environmental and genetic factors is important for cancer development. Carcinogenesis is a slow and complex process, during which there is progressive accumulation of genetic and epigenetic alterations in cells, contributing to a malignant phenotype [6]. Major modifications derive from the occurrence of mutations in oncogenes and tumor suppressor genes in normal cells, triggering an uncontrolled cell growth by their activation (oncogenes) and/or inactivation (tumor suppressor genes), causing deregulation of several crucial pathways [7]. The carcinogenesis process is divided in some main steps: transformation (consisting of initiation and promotion), growth and progression (characterized by local invasion followed by metastization). In the first step, a cell must suffer genomic alterations caused by carcinogenic agents (chemical, physical or biological). A sequence of multiple alterations modifies key processes in the cell, such as proliferation and apoptosis, conferring a neoplastic phenotype to the cell, which provides selective advantage [7-9]. During cancer development, associated with genomic instability and an incorrect DNA repair, cells acquire autonomous and limitless growth capacity, insensibility to apoptotic and growth inhibitory signals and capacity of evasion from the immune system. Additionally, an inflammatory environment is promoted and the cellular energy metabolism is reprogrammed. All these factors, together with induction of angiogenesis and activation of invasion and metastasis, culminate in cancer progression and systemic dissemination of the cancer to other organs [8, 9].

Concomitantly with the malignancy of the tumor cells, the microenvironment in which cancer cells are involved also has a pivotal role in the pathophysiology of a tumor. Recently, the components of the milieu surrounding cancer cells have been studied, revealing their importance in the maintenance and expansion of the tumor, as well as in drug resistance (**Figure 1**) [10-12]. The relevance of the tumor microenvironment has recently been accepted and contrasts with the older studies that only focused on the biology of cancer cells without taking into account their surroundings [12]. In fact, endothelial cells, pericytes, cancer associated fibroblasts (CAFs), bone marrow progenitor cells, macrophages and cancer stem cells continuously interact with neoplastic cells, by sending external signals to each other. These cells, in association with the extracellular matrix and soluble secretion factors (e.g. growth factors) constitute the tumor microenvironment [13, 14]. Evidence suggest that targeting components of the tumor microenvironment could be a complement approach to current treatments, improving clinical outcomes and providing an extra level in therapeutic intervention [12, 15].

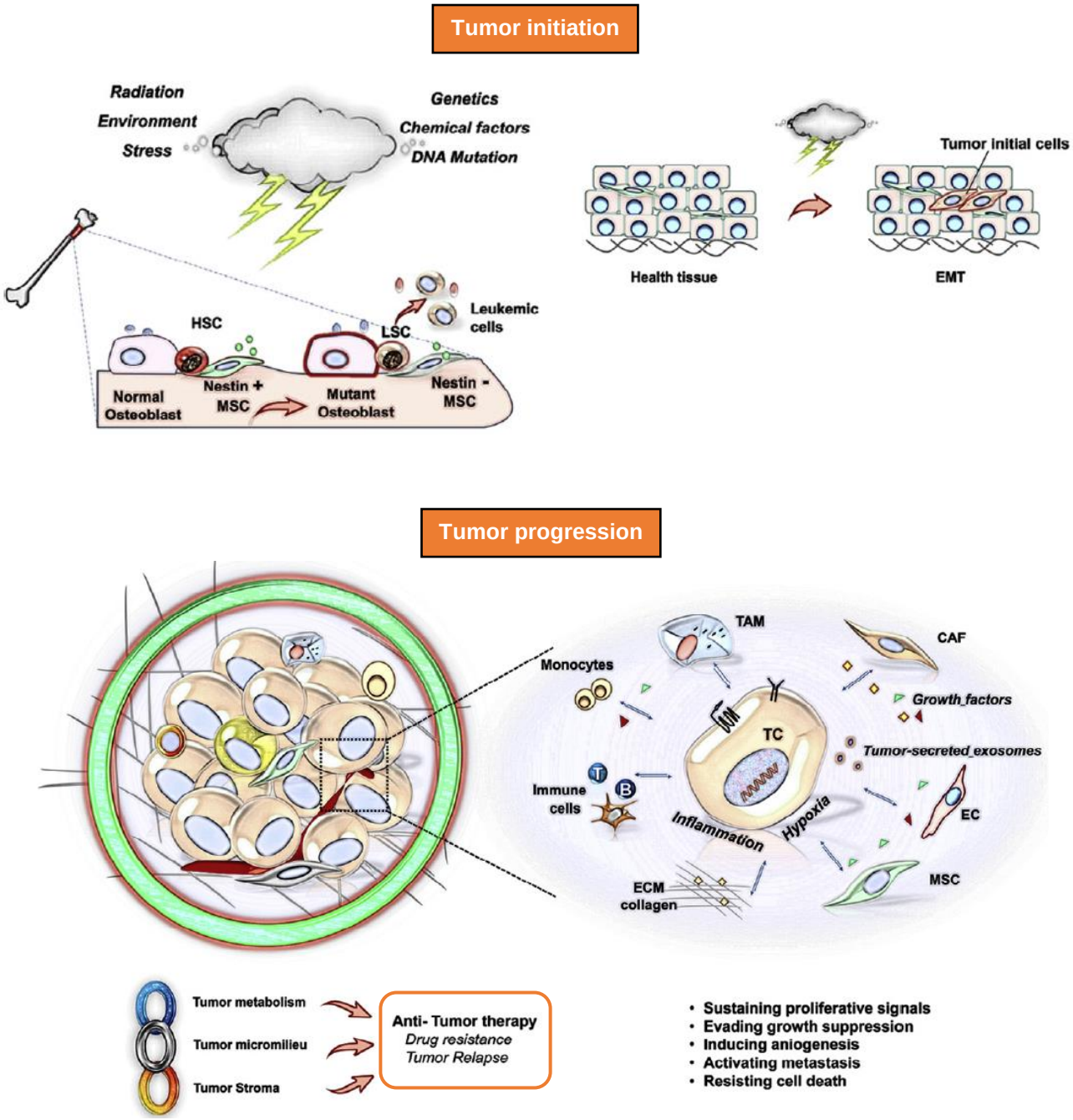


Figure 1. Importance of the tumor microenvironment during tumorigenesis. HSC – Hematopoietic Stem Cells; MSC – Mesenchymal Stem Cells; LSC – Leukemia Stem Cells; EMT – Epithelial-mesenchymal Transition; TAM – Tumor-associated Macrophages; CAF – Cancer-associated Fibroblasts; EC – Endothelial Cell; ECM – Extracellular Matrix; TC – Tumor Cell (adapted from [12]).

1.2. Chronic Myeloid Leukemia (CML)

1.2.1. Definition, molecular events and epidemiology

Chronic myeloid leukemia (CML) is a hematological disease disturbing the myeloid (mostly granulocytic) compartment of the hematopoietic stem cell system. In 90 – 95% of the cases, this myeloproliferative disorder occurs from a reciprocal translocation between the BCR (Breakpoint Cluster Region) and ABL (Abelson) genes, on chromosomes 22 and 9, respectively. Such chromosomal aberration, t(9;22)(q34; q11), originates the Philadelphia chromosome (Ph), which in turn generates the BCR-ABL oncoprotein [16-20]. This protein is a tyrosine kinase that leads to several alterations in critical mechanisms of the cell, by deregulation of important downstream pathways. Indeed, BCR-ABL acts on various substrates, such as the nuclear factor kappa B (NF-kB), Ras, mitogen activated protein kinase (MAPK), signal transducers and activators of transcription (STAT), c-jun N-terminal kinase (JNK), phosphoinositide 3- (PI-3) kinase, stress-activated protein kinase (SAPK), and c-Myc. Hence, the constitutive activation of BCR-ABL drives changes in different cellular processes such as cell growth, differentiation, adhesion and apoptosis. The stimulation of these pathways leads to an independent proliferation associated with increased resistance to apoptosis (**Figure 2**) [21-26].

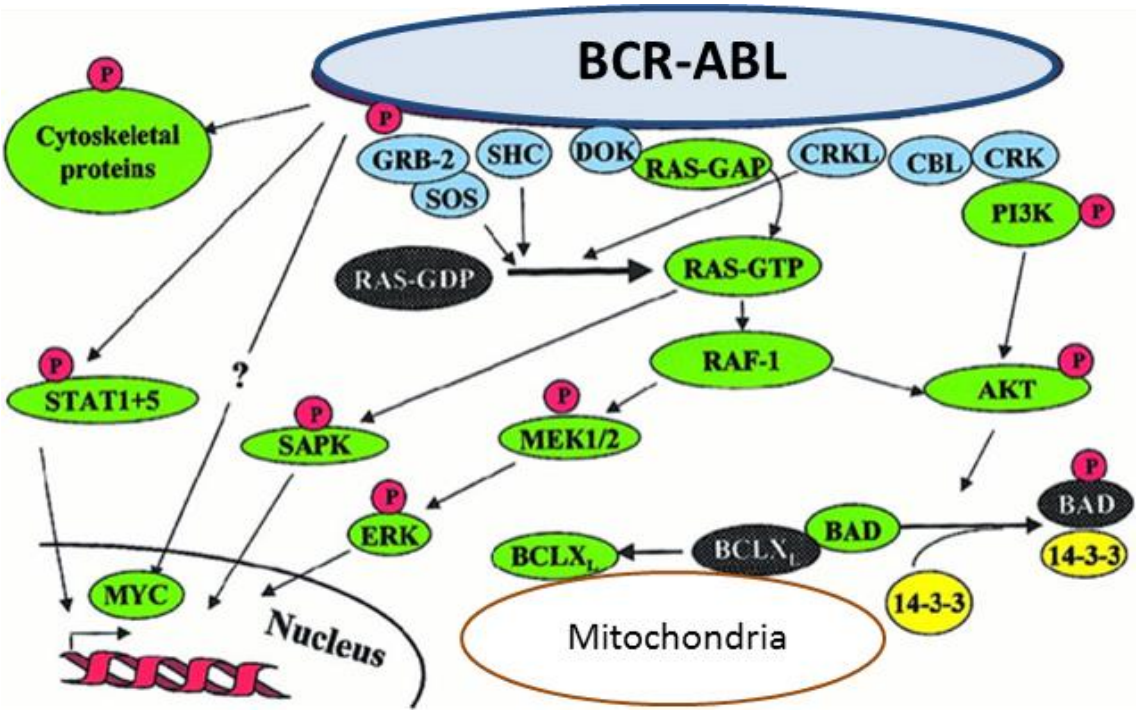


Figure 2. Cascade of some downstream signal-transduction pathways affected by BCR-ABL. The deregulation of these pathways, by the constitutively activation of the BCR-ABL oncoprotein, alters crucial mechanisms in the cell, culminating in chronic myeloid leukemia. P indicates phosphate (adapted from [21]).

The worldwide incidence of CML is of 0.6 to 2.0 cases per 100 000 individuals. Thus, CML is a relatively rare pathology and it is most common in men. At diagnosis, the median age is 57 to 60 years (data from European CML registries), but it may be diagnosed in all age groups, even though with low frequency in young people [27, 28]. There is no association of this disease with race or ethnicity, nor with geographic or genetic predisposition; however, it is thought that ionization radiation in high doses is associated with CML. Among all leukemia cases, about 15% of them are of CML. Despite scarce reliable data from poor countries, it is estimated that 100 000 patients will be affected every year by CML globally, representing a significant worldwide health burden [28, 29].

CML malignancy develops in three stages: chronic phase, accelerated phase and blast crisis. The first phase, in which 90% of the patients are diagnosed, is characterized by the excess of myeloid cells that retain the capacity to differentiate and to have normal function. Patients may remain approximately 3 to 8 years in this phase. When the disease progresses to the next phase, a gradual loss of differentiation and an accumulation of blasts occur. Lastly, the natural course of the disease culminates in the blast crisis, characterized by the presence of 20% or more blasts in the bloodstream or bone marrow, or by infiltration of blasts extramedullary or in the bone marrow compartment. This phase has an acute leukemia behavior, fast progression and extremely short survival (reduced to few months). The accumulation of molecular anomalies may drive the increasing loss of the ability of leukemic cells to differentiate [18, 30]. Of note, normal and leukemic hematopoiesis have a common origin, since progenitor cells may undergo transforming mutations becoming a tumorigenic progenitor cell (**Figure 3**).

The clinical presentations are frequently granulocytosis (higher amount of leukocytes, mainly granulocytes, in the blood), hypercellularity of the bone marrow and splenomegaly. Nevertheless, approximately 40% of patients do not have symptoms and CML is often diagnosed based on abnormal blood cell counts, found in a blood test performed with another purpose [16]. When CML is detected, symptoms are often mild at the beginning and become slowly more aggressive. Some of the symptoms are: tiredness, fever, sweating excessively, weight loss, early satiety, itching, bleeding, bone pain and abdominal swelling or discomfort caused by an enlarged spleen. Agents such as hydroxyurea and interferon-α (IFNα) can be used to control the symptoms, even though they are not able to cure the patients. The blast crisis is non-responsive to therapies and culminates irrevocably in death [31].

The usual procedures used to diagnose CML are: physical exam (pulse and blood pressure check, lymph nodes and examination of abdominal abnormalities); blood tests

(complete blood count); bone marrow tests (collection of bone marrow from hipbone, by biopsy or aspiration); tests for searching the Ph chromosome or the *BCR-ABL* gene (analysis of blood or bone marrow samples by specific tests, such as polymerase chain reaction – PCR, and fluorescence in situ hybridization – FISH) [32].

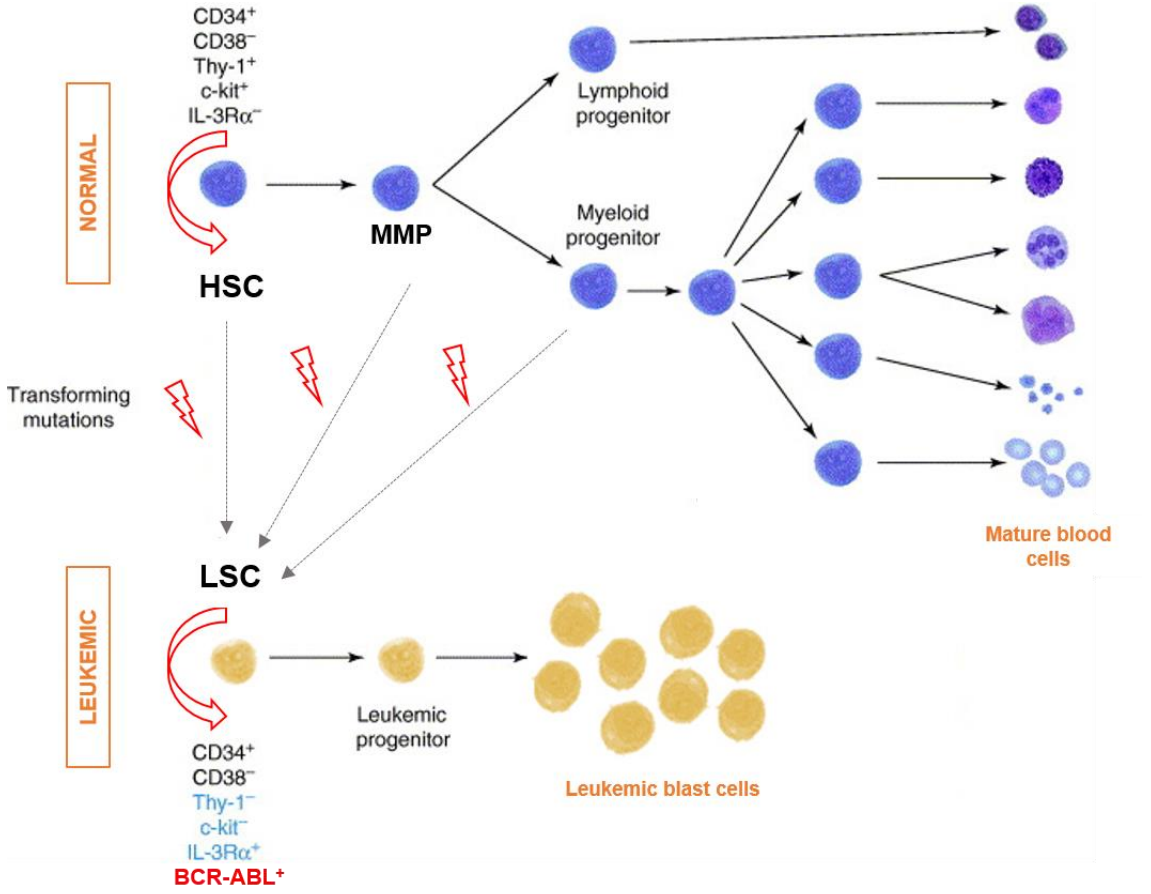


Figure 3. Schematization of the normal and leukemic hematopoietic hierarchies. A small population of self-renewing cells, the hematopoietic stem cells (HSC), sustains and originates lineage-restricted cells, with progressively differentiation and reduced self-renewal ability, producing mature blood cells. By the acquisition of mutagenic events, leukemic stem cells (LSC) may arise either from HSC, multipotent progenitors (MPP) or committed progenitor cells [33] (however, this topic remains controversial since some authors defend that only HSC could give origin LSC [34]). LSC share some features with normal HSC, driving CML progression, but having an altered differentiation program, as shown by the aberrant expression of several cell-surface markers (indicated in blue) and by the expression of the oncogene *BCR-ABL*. (Adapted from [33] and from [35])

1.2.2. Targeted drugs to treat CML

Prior to the use of BCR-ABL targeted therapies, some other options had been used in the management of CML. In the beginning of 20th century, an arsenic solution was the first treatment for CML. Other treatment options then followed such as: spleen irradiation and the use of busulfan, hydroxyurea and cytosine arabinoside (cytarabine). Nonetheless, they presented high toxicity and, in some cases, although they provided improvements in terms of decreased symptoms, it did not prolong patients' lives [36, 37].

Interferon-α (IFNα), a nonspecific immunostimulant, was the first drug found to have the capacity of extending the first stage of the disease and delaying the progression to the accelerated phase. IFNα acts by regulating T-cell activity and provides encouraging responses, namely complete hematologic response (CHR) and complete cytogenetic response (CCyR) – which definition is clarified in the next section – thereby prolonging the median survival. Nevertheless, due to the lack of specificity, toxicities arose and secondary symptoms appeared, leading to the discontinuation of this therapy [38, 39].

Allogeneic stem cell transplantation provides long-term outcomes, being the only curative therapy proven so far. Still, it has been associated with high morbidity and mortality, and it is difficult to find available matched donors. Moreover, it is known that quiescent CML stem cells persist after transplantation, highlighting the need to find effective alternatives [40-43].

1.2.2.1. Imatinib mesylate (STI571)

The BCR-ABL tyrosine kinase activity is a key factor for CML pathophysiology [44]. Once identified, this molecule became a potential target for developing a selective inhibitor. Lydon and colleagues designed, in 1996, various compounds which inhibit the BCR-ABL domain. Among all compounds tested, they found the 2-phenylaminopyrimidine, which gave rise to imatinib mesylate (also called STI571, CGP57148B or Gleevec®, from Novartis Pharmaceuticals) [45-47].

STI571 (**Figure 4**) was highly effective in targeting cells expressing BCR-ABL protein. The drug was also selective for platelet-derived growth factor receptor (PDGF-R) and for the c-kit receptor. STI571 was first used in phase I clinical trials in 1998, 38 years after the first description of the Ph chromosome. Due to the undoubtedly satisfactory results,

it continued to phase II and III clinical trials, being approved in 2001 by the Food and Drug Administration (FDA) [46-52].

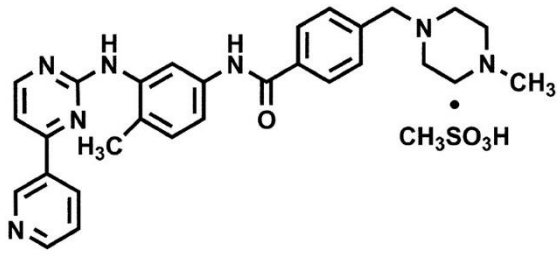


Figure 4. Imatinib's molecular structure.

The tyrosine kinase inhibitor (TKI) STI571 binds to the catalytic pocket of the BCR-ABL protein tyrosine kinase, competing with ATP for this site. In this way, the autophosphorylation of BCR-ABL and the phosphorylation of its substrates is inhibited, preventing the activation of other effector molecules [25, 44, 47, 53, 54].

The standard regimen of STI571 doses consists of 400 mg daily (adults) or 260-340 mg/m^2/day (children) [55]. STI571 is highly effective and better tolerated than the previous therapies, having strongly improved the quality of life of these patients. However, in some cases, patients have a refractory disease, relapse or are intolerant to STI571, leading to treatment discontinuation[23]. Therefore, it was necessary to develop second generation TKIs that could overcome STI571 resistance [56-58].

Nilotinib (Tasigna®, from Novartis Pharmaceutical) and Dasatinib (Sprycel®, from Bristol-Myers Squibb) were developed to treat patients who did not response to first line treatment with STI571. These drugs are much more potent against BCR-ABL than STI571 and about 50% of patients who previously showed resistance to STI571 will reach a complete cytogenetic remission. Nonetheless, these drugs are not effective against the most common mutations in the BCR-ABL domain, particularly the T315I mutation, known to cause resistance to these drugs [59-62]. Hence, various other TKIs have been found to overcome new cases of resistance, such as Bosutinib (Bosulif®, from Wyeth) and Ponatinib (Iclusig®, from ARIAD Pharmaceuticals). This last drug is able to inhibit the most common type of mutation in BCR-ABL which is responsible for most cases of drug resistance, the T315I mutation [63-65].

1.2.3. Resistance to BCR-ABL tyrosine kinase inhibitors (TKIs)

Development of various drugs targeting BCR-ABL, known to have a central role in CML pathogenesis, represented a huge improvement in CML patients' clinical outcomes. Nonetheless, even though TKIs were effective in approximately 80% of CML cases, it was found that 20% of the patients do not respond to the first generation drugs[66].

First, it is important to understand how therapeutic response is monitored in CML. In a simplified way, the criteria established for successful response to therapy include:

- Complete Hematologic Remission (CHR): characterized by normal blood cell count and total disappearance of symptoms of the disease;
- Complete Cytogenetic Response (CCyR): meaning the total absence of Ph^+ metaphases (in a total of 20 bone marrow metaphases analyzed);
- Complete molecular response (CMR), with no detection of BCR-ABL transcripts (this however depends on the sensitivity of the test, therefore there is no globally accepted definition for CMR) [67].

According to this, definition of resistance to STI571 consists of incapacity to accomplish any of the criteria: CHR at 3 months, any CyR at 6 months, partial CyR at 12 months or CCyR at 18 months of STI571-treatment [68].

Cancer drug resistance may be intrinsic, caused by preexisting mechanisms, whereby malignant cells are resistant to therapies even before treatment initiation [69, 70]. This is also known as primary resistance and it is the cause of < 10% of TKIs resistance cases, occurring during chronic phase [71]. Alternatively, drug resistance can be acquired during treatment, due to mechanisms acquired during the "selective pressure" caused by the drug [69, 70]. This acquired resistance is also called secondary resistance and it is drug induced, occurring mostly in CML late stages [71-73]. The molecular procedures behind this resistance are divided into BCR-ABL-dependent and independent mechanisms [26, 74].

A deep understanding of the molecular mechanisms responsible for drug resistance of CML patients is essential in order to implement the most adequate therapies and search for novel and alternative drugs.

1.2.3.1. BCR-ABL-dependent mechanisms

1.2.3.1.1. BCR-ABL mutations

The occurrence of point mutations in the BCR-ABL kinase domain is the most common mechanism responsible for TKIs resistance. These mutations, occurring in residues that direct bind to STI571, generate conformational alterations of BCR-ABL therefore decreasing the affinity of TKIs to the BCR-ABL domain [56, 75].

More than 100 mutations were identified until now, varying in clinical relevance [75]. The T315I (Th315→Ile315) mutation was the first reported point mutation that confers resistance to TKIs and is the most common among cases of patients with resistance. The genetic region affected codes for the ATP-binding site of the ABL domain and results in a substitution of a threonine (Thr) by an isoleucine (Ile) at amino acid 315. This "gatekeeper" mutation prevents the hydrogen bond to be formed between the oxygen of the side chain of threonine 315 and the NH_2 group of STI571. Indeed, isoleucine has a hydrocarbon extra group, inhibiting STI571 binding [76]. The T315I mutation confers resistance to all BCR-ABL inhibitors approved so far, except to Ponatinib [65].

There are other relevant mutations which cause TKIs' resistance, mapped in different sites of the kinase domain. The other most common mutations are the E255K (located in the P-loop) and the H396P (located in the activation loop), which are conserved and flexible loops, respectively. The P-loop (phosphate- or ATP-binding loop) forms the top of the active site of BCR-ABL and is responsible for coordinating the phosphate group of the ATP molecule (**Figure 5**). The activation loop acts as a backbone of the binding substrate, so it can be phosphorylated. By destabilizing the desired conformation for binding to STI571 and other BCR-ABL inhibitors, these mutations represent an important mechanism of drug resistance, associated with bad prognosis [47, 77-79].

Evidence suggests that it is not the drug treatment that induces such mutations. Instead, it is believed that these mutations are already present within some of the tumor cells (due to tumor heterogeneity) conferring advantage to those cells when under the presence of the drug, by selective selection. In other words, these rare mutant clones pre-exist in the tumor and have the ability to survive and proliferate even under treatment with these drugs, gradually taking over the drug-sensitive cells [80].

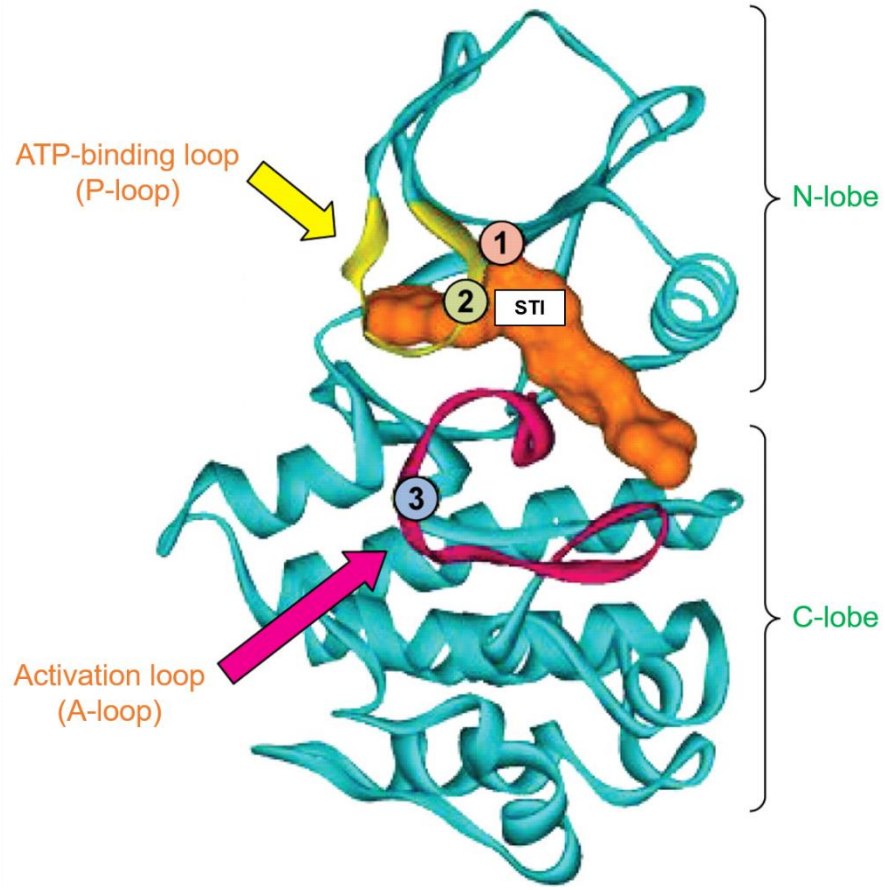


Figure 5. Ribbon illustration of ABL complexed with Imatinib. Imatinib (STI, in orange) occupies the ATP-binding site (P-loop) of ABL protein (blue) when the A-loop is in the closed configuration. The relative positions of three of the most frequently mutated residues are also represented: 1 – T315I, in the gateway position; 2 – E255K, in the P-loop; and 3 – H396P, in the activation loop (adapted from [44])

1.2.3.1.2. *BCR-ABL* amplification

The amplification of the *BCR-ABL* fusion gene and, consequently, the overexpression of the BCR-ABL oncoprotein may also provide relative resistance to TKIs. The resistance occurs because of the higher quantity of the target protein which is needed to be blocked by a therapeutically acceptable dose [81, 82]. This mechanism of resistance represents about 18% of the cases of STI571 treatment failure (against approximately 50% of resistance cases due to point mutations, referred above). It was shown that the amplification of the gene decrease after discontinuation of the treatment for some weeks, suggesting that continuous STI571 administration may cause a selection of cells with increased copies of *BCR-ABL* [76].

Nevertheless, a considerable proportion of cases of TKIs resistance is not caused by BCR-ABL overexpression, neither by point mutations in the kinase domain (indeed,

some of the mutations detected do not cause loss of sensitivity to STI571). Thereby, drug resistance might be caused by other mechanisms, non-dependent of BCR-ABL [74].

1.2.3.2. BCR-ABL-independent mechanisms

1.2.3.2.1. Overexpression of drug-efflux proteins

ATP-binding cassette – ABC – transporters are a superfamily of transmembrane proteins that were found to cause the efflux substrate drugs from the cell, leading to a decrease on the drugs intracellular concentrations, thus promoting chemoresistance [69, 83]. Multidrug resistance (MDR) is a phenomenon corresponding to the development of concomitant resistance to various drugs, independently of their mechanism of action or chemical structures. MDR is frequently associated with overexpression of these ATP-dependent pumps [84].

Some *BCR-ABL*$^{+}$ cell lines resistant to STI571 and CML stem cells were described to overexpressed ABC proteins, such as P-glycoprotein (P-gp, ABCB1 or MDR1) and the ATP-binding cassette sub-family G member 2 (ABCG2 or BCRP). Of note, STI571 is a substrate for these proteins. So, it is accepted that drug-efflux pumps are responsible for some cases of STI571 resistance, by decreasing the bioavailability of the drug inside of the cell [85-91].

1.2.3.2.2. Activation of alternative signaling pathways

The cellular dependence on the original drug target (BCR-ABL) can be alleviated by the permanently activation of BCR-ABL downstream signaling pathways, such as the kinases of the Src family. These kinases keep working even in the presence of STI571, suggesting that they might contribute to a BCR-ABL-independent mechanism of resistance. On the other hand, the Src family of kinases may phosphorylate the SH2 and SH3 (Src-homology-2 and -3) domains of BCR-ABL, leading to an increase in the BCR-ABL kinase activity and therefore altering the cellular susceptibility to STI571. Several pathways of intracellular signal transduction are modulated by the Src family, such as those involved in survival, cell growth and tumor dissemination. Therefore, therapies based on anti-Src family of kinases, simultaneously targeting BCR-ABL, could be a powerful tool to overcome some cases of drug resistance. In fact, the combination of dual inhibitors (e.g. dasatinib and

bosutinib) suggested that it was possible to counteract cases of STI571 resistance, except for the cases due to T315I BCR-ABL mutant [54, 74, 92].

The p53 pathway also has an important role in response to STI571 therapy. p53 inactivation (often associated with CML progression) blocks, both *in vitro* and *in vivo*, cellular response to this drug. Therefore, mutations that affect the p53 pathway may contribute to STI571 resistance in later phases of CML [93].

Moreover, autocrine loops might also contribute to resistance to STI571. Indeed, interleukin-3 and granulocyte-colony stimulating factor (G-CSF) were produced by CD34+ primitive cells in patients in the chronic phase of the disease. Such cytokines promote the autocrine stimulation of cellular proliferation events (by pathways such as JAK/STAT and Ras/Raf/Mek pathways), therefore preventing STI571-induced apoptosis [94].

Besides, other mechanisms have been further reported, with relevant contribution to drug resistance and consequently to treatment failure. For instance, the presence of a quiescent population of primitive *BCR-ABL*$^{+}$ stem cells is a major cause responsible for relapses. This population of stem cells is preserved in bone marrow niches and, when treatment with TKIs is discontinued, they may spontaneously leave the G_0 phase and acquire a state of constitutive proliferation [94-96]. In addition, point mutations outside the BCR-ABL kinase domain have also been described in cases of drug resistance, often in positions essential for the maintenance of the inactive state of the oncoprotein (such as in N-terminal region and the SH2 and SH3 domains) [56]. Finally, the under-expression of drug uptake transporters, such as the human organic cation transporter 1 (hOCT1), may also decrease the intracellular STI571 concentration [97, 98].

Therefore, BCR-ABL-independent mechanisms have a great impact in CML progression.

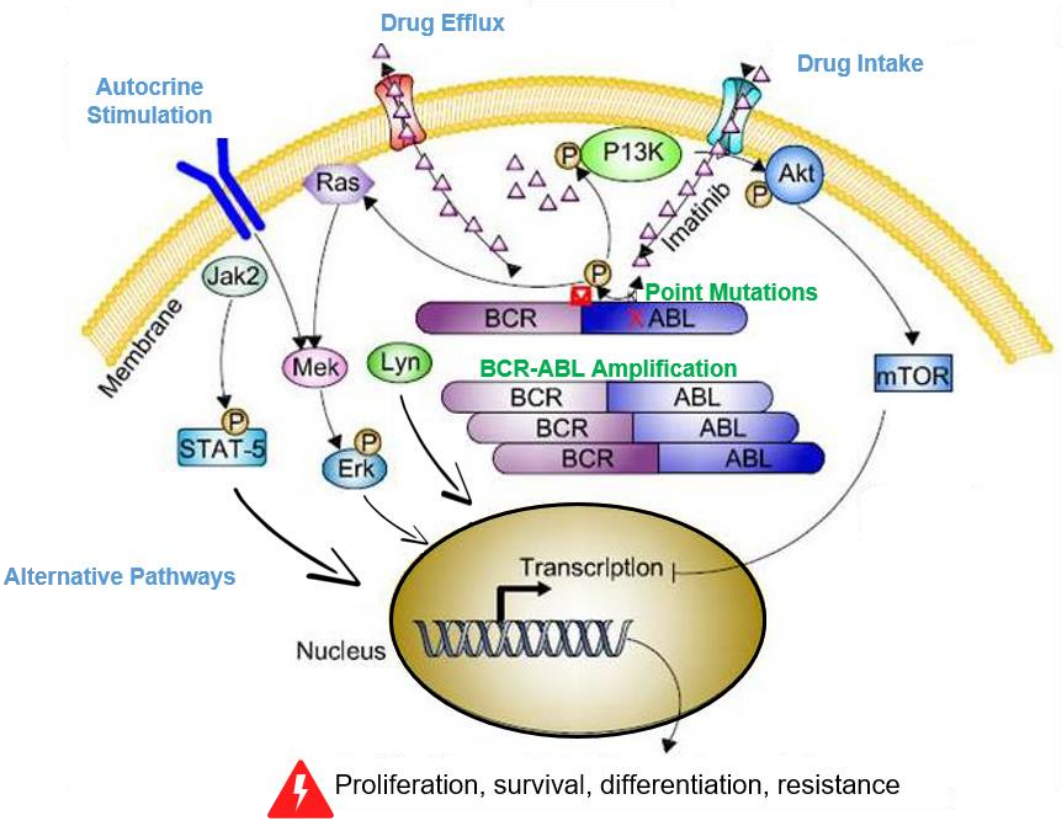


Figure 6. Summary of several mechanisms of resistance against tyrosine kinase inhibitors. BCR-ABL-dependent and -independent mechanisms are depicted in green and blue, respectively. (Adapted from [26])

1.3. Transfer of drug resistant traits by Extracellular Vesicles (EVs)

1.3.1. Exosomes and microvesicles: biogenesis, release by donor cells and uptake by recipient cells

The complex interactions occurring in the tumor microenvironment are maintained by a constant influx and efflux of biological material through the cellular membrane. In addition to active and passive transport, there are other mechanisms through which cells communicate with the outside environment (with the microenvironment of the cells or with distant sites). Intra- and intercellular communication between neoplastic and non-neoplastic cells is achieved by cell-cell interactions (gap junction-mediated), paracrine mechanisms (growth factors, chemokines and proteases) as well as by extracellular vesicles (EVs). It is believed that communication mediated by EVs provides a mechanism by which more complex information can be exchanged between cells [99-104].

EVs are small structures but variable in size (10 – 1000 nm), surrounded by a lipid bilayer and produced by all types of cells, prokaryotic and eukaryotic, under physiological or pathological conditions. They have been isolated from several biological fluids such as blood, semen, breast milk, urine, saliva, bile, amniotic fluid and cerebrospinal fluid. These nanoparticles are important mediators of intercellular communication. EVs are mainly exosomes, microvesicles and apoptotic bodies. The exosomes are the smallest, with a size range between 30 – 100 nm and with an endosomal origin. They are formed within endosomes, which are known to pass through different stages. The late endosomes, known as multivesicular bodies, culminate in lysosome fusion (with destruction of their content) or fusion with the plasmatic membrane followed by release of exosomes from the cell. On the other hand, the microvesicles are larger in relation to exosomes (50 – 1000 nm) and are generated by direct budding of the plasma membrane, resulting from a dynamic interchange between contraction of cytoskeletal proteins and redistribution of phospholipids. Finally, apoptotic bodies are the largest vesicles (from 500 to 4000 nm) and are released during apoptosis due to membrane blebbing [105-108].

Efforts have been done in order to discover proteins present in EVs that allow distinguishing between microvesicles and exosomes. It was found that exosomes contain heat-shock proteins (HSP70 and HSP90), tetraspanins (including CD9, CD63 and CD81) and proteins involved in immunologic functions, like molecules of the major histocompatibility complex class II. Nevertheless, recent research showed that these proteins are also present in cell pellets enriched with larger EVs. Therefore, it is difficult to find reliable specific markers of exosomes versus microvesicles. Indeed, this is a relatively recent field of research and standardization of nomenclature and protocols need to be optimized [106, 109, 110].

Regarding their release and uptake mechanisms, it is believed that EVs shed by donor cells might interact only with recipient cells that they specifically recognize. However, further work needs to be done to fully understand this phenomenon. The uptake of EVs by recipient cells may occur either by: i) docking at the plasma membrane of the recipient cell, ii) direct fusing with the plasma membrane of that cell (thereby delivering the cargo of the EVs into the membrane or the cytosol of the recipient cell), or iii) by endocytosis (thus fusing the EVs with an endocytic organelle) [100, 105, 106].

EVs released by tumor cells contain biological material from the donor cells. The transfer of their cargo to recipient cells may contribute to tumor growth and metastasis [111-116].

The EVs cargo may include microRNAs (miRNAs), mRNAs or other types of RNA (such long noncoding RNAs lncRNAs), proteins (mainly the ones involved in vesicles formation, but also others such as oncoproteins or drug efflux pumps), lipids (such as phospholipids, glycerophospholipids, sphingolipids) and even small pieces of genomic DNA [117, 118].

Some studies have suggested that cancer cells produce more EVs than normal cells. Furthermore, it has been demonstrated that the quantity of shed vesicles changes during cancer development and their cargoes are different in tumors with high or low metastatic potential [119-121].

Emerging evidence supports the possibility that EVs might become a good source of biomarkers of disease, considering their content in specific molecules from the donor cell, thus reflecting the cell of origin [101, 122-124].

Interestingly, alternative therapies based on EVs have been proposed. Therapies proposed so far include activation of the immune system by dendritic cell-derived exosome-based vaccines; the use of EVs for transport and delivery of drugs, miRNAs or siRNAs to targeted cells; and the selective removal of EVs released by cancer cells, from the blood and other body fluids, by a process similar to hemodialysis [113, 125-128].

1.3.2. Role of EVs in tumorigenesis and drug resistance

EVs are important players on tumor development and resistance. For example, it is believed that EVs released by cancer cells or by various cells that compose the tumor microenvironment may influence tumor heterogeneity. Additionally, EVs may modulate the immune response (enabling immune evasion of the tumor cells and contributing to initiating the inflammatory response) or participate in the differentiation of CAFs (transporting growth factors, such as TGF-β1 and FGF-2) and transport the bioactive molecules produced by them (such as interleukin-6 and prostaglandins). In addition, EVs can trigger angiogenesis and help in the metastatic process, by inducing the epithelial to mesenchymal transition or by preparing the pre-metastatic niches. Moreover, EVs also take part in some cancer drug resistance (CDR) processes, being described as a remarkable vehicle of CDR dissemination [110-112, 116, 123, 126, 129, 130]. Indeed, EVs released from donor drug-resistant cells may horizontally transfer mediators of CDR to recipient drug-sensitive cells. Afterwards, the recipient cells may acquire the drug-resistant phenotype by incorporating the cargo of such vesicles. The cargo of EVs which may mediate CDR spreading might include miRNAs, lncRNAs or drug-efflux pumps (**Figure 7**) [111, 117].

The miRNAs may be encapsulated into EVs, conferring them high stability and protection from the action of RNases. In addition, miRs contained in EVs may function as signaling molecules that influence the targeted cells phenotype [131]. In 2012, Jaiswal R. and his collaborators analyzed, in hematological and non-hematological models, the miRNA profiles of drug-sensitive recipient cells, after co-culture with EVs derived from drug-resistant cells. They found that there was a transfer of CDR traits into recipient cells, mediated by selective packaging of miRNAs into those EVs by the donor cells [132].

Although the role of lncRNAs in CDR is not clearly understood, studies have reported the enrichment of these RNAs in EVs, together with an increase in their expression in the donor cells after chemotherapy treatment. Besides, a study in which EVs derived from hepatocellular cancer cells, after treatment with several chemotherapeutic agents (with consequent enrichment in lincRNA-VLDR), were co-cultured with recipient cells, showed an upregulation of a lncRNA in the recipient cells and a reduction in apoptosis induced by chemotherapy [133, 134].

The presence of drug efflux pumps in the cargo of EVs has also been found. As mentioned above, those proteins are involved in the efflux of drugs, thereby decreasing the concentration of substrate drugs to sublethal levels. It was shown that these pumps may be transferred by EVs to recipient cells, providing a mechanism of intracellular transfer of multidrug resistance [135, 136].

Other type of molecules carried in EVs shed by drug-resistant cells may also mediate propagation of CDR traits, by inducing EVs production or regulate other mediators of drug resistance. An example is the mRNA encoding inhibitors of apoptosis proteins (IAPs), found to be present in EVs from CML cell lines. Incubation of drug-sensitive cells with EVs derived from drug-resistant cells promoted the accumulation of P-gp and miRNAs, as well as IAPs in recipient cells. The resistance then exhibited by the recipient cells was proposed to be a result of the transfer of all the three mediators.

Interestingly, there is controversy regarding the possibility of selectivity for the recipient cells of EVs. Indeed, some recent studies indicated that EVs may have “tissue selectivity”. In one of those studies, breast cancer cells overexpressing P-glycoprotein (P-gp) transferred this protein (via EVs) only to malignant recipient cells. However, the same study showed that leukemia cells released EVs that transferred P-gp and multidrug resistance-associated protein 1 (MRP-1), to both malignant and non-malignant cells [137]. In another study, CML cells exhibited no selectivity associated to the cell type of recipient cells, since EVs from drug-resistant CML cells transferred P-gp to drug-sensitive cells of both breast and lung cancer cells [138]. Taken together, such findings suggest that further

research is needed in order to understand if there is selectivity on the process of intercellular transfer of EVs cargo.

In summary, the horizontal transfer of drug resistance mediated by EVs has recently been discovered as a relevant mechanism of propagation of a CDR phenotype. Indeed, it was shown that not only the mediators of drug resistance are transferred into recipient cells, through EVs shed by donor drug-resistant cells, but also that they are functional in the recipient cells [117].

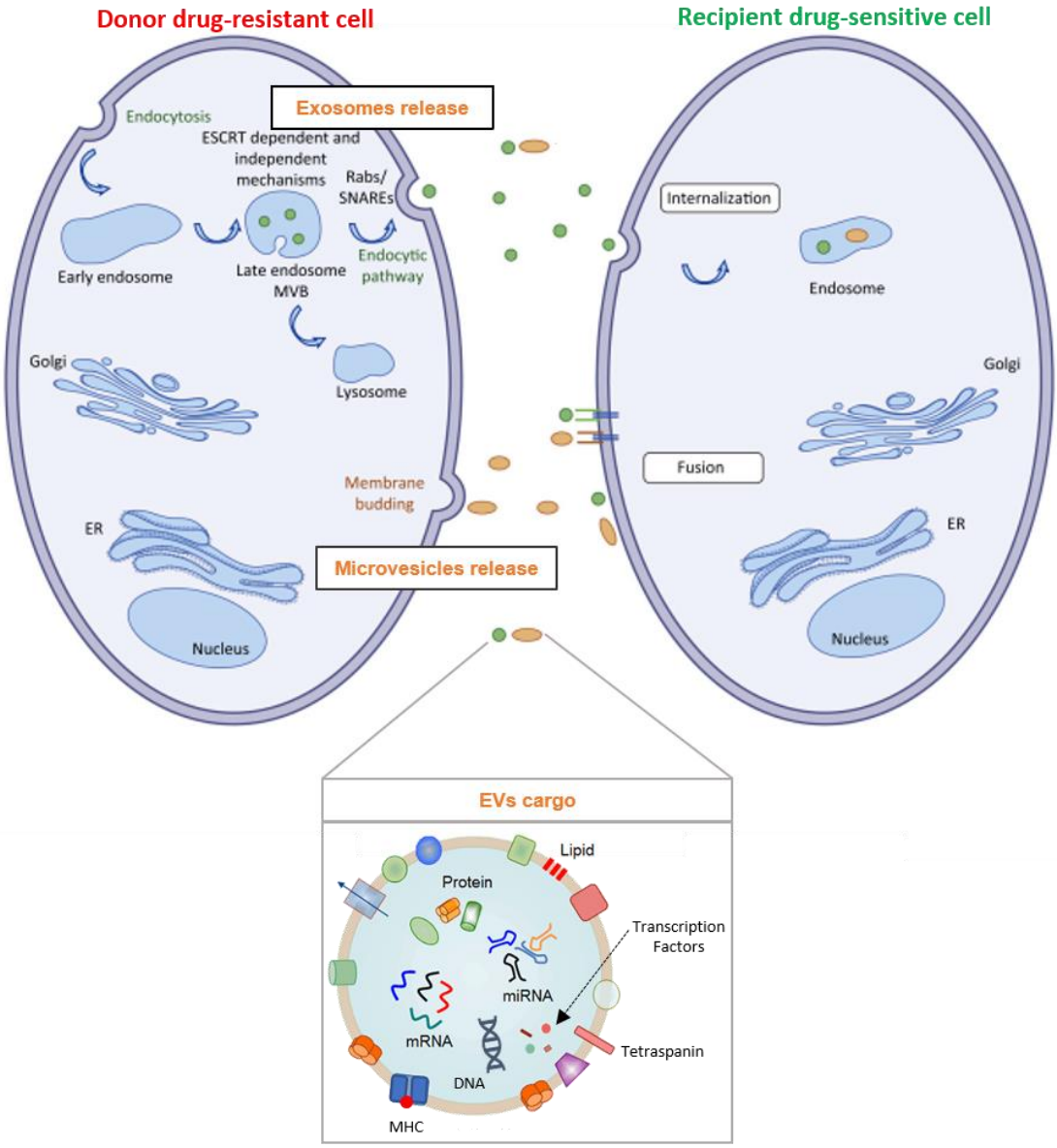


Figure 7 Intercellular transfer of drug resistance mediated by extracellular vesicles. These vesicles are involved in intercellular communication, particularly the microvesicles (which emerge from direct budding of the plasma membrane of the cell) and exosomes (with endocytic origin, formed by inner sprouting of the multivesicular body, MVB, membrane). Drug resistance transfer may occur

from drug-resistant cells (donor of EVs) to drug-sensitive cells (recipient of EVs) mediated by extracellular vesicles (EVs). The cargo of the EVs may include, among other components, proteins (such as drug-efflux pumps), miRNAs and long noncoding RNAs (lncRNAs), responsible for cancer drug resistance dissemination. Once the recipient cell is reached, EVs may be internalized by endocytosis or may fuse with the cellular plasma membrane. Small green vesicles represent exosomes and bigger brown vesicles represent microvesicles. ESCRT – Endosomal Sorting Complex Required for Transport; Rabs – Ras-associated binding proteins; SNAREs – Soluble *N*-ethylmaleimide-sensitive factor Activating protein Receptor; ER – endoplasmic reticulum; mRNA – messenger RNA; miRNA – micro RNA; MHC – major histocompatibility complex. (adapted from [117])

Many questions in this recent field of research still have no answer but it is expected that, in the near future, clinical data will elucidate the real impact of this type of CDR transfer in patients' response to treatments and clinical outcome [117].

1.4. Aims

1.4.1. Main aim

The overall aim of the present work is to investigate if:

i) EVs released by BCR-ABL$^+$ chronic myeloid leukemia cells had BCR-ABL on their cargo; and ii) EVs shed by a drug-resistant BCR-ABL$^+$ cell line (KBM5-STI, harboring an additional point mutation in *BCR-ABL,* which confers resistance to STI571) were responsible for intercellular transfer of drug-resistance to their drug-sensitive counterpart cells (KBM5).

1.4.2. Specific aims

The specific aims of the present work were to:

i. Characterize the pair of counterpart cell lines, KBM5 and KBM5-STI, regarding response to STI571 in order to confirm their drug sensitive and resistant phenotypes, respectively;

ii. Isolate EVs from those cell lines and characterize them according to their size, morphology and protein content;

iii. Investigate if EVs released by these *BCR-ABL*$^+$ leukemia cell lines carry BCR-ABL (in terms of protein and mRNA) on their cargo;

iv. Verify if drug resistance could be transferred from leukemic drug resistant cells (with mutant *BCR-ABL*) to leukemic drug sensitive cells (with wild-type *BCR-ABL*).

II. Materials and Methods

2.1. Cell culture

The human chronic myeloid leukemia (CML) cell lines, KBM5 (sensitive to imatinib) and its *BCR-ABL* mutated counterpart cell line KBM5-STI (resistant to imatinib and generated from the parental KBM5 cell line), were kindly provided by Dr. Miloslav Beran and Dr. Bing Z. Carter from the University of Texas M.D. Andersen Cancer Center, Houston, Texas [139, 140].

KBM5 and KBM5-STI cell lines grow in suspension and were routinely grown in RPMI-1640 medium, with Ultraglutamine I and 25 mM HEPES (Lonza), supplemented with 10% fetal bovine serum (FBS, from Biowest) at 37 ºC in a humidified atmosphere containing 5% CO_2. The KBM5-STI resistant cell line was maintained in culture in the presence of 1 μM imatinib mesylate, STI571 (Gleevec®, from Sigma-Aldrich, Co.), by addition of the drug to the medium following each passage. Cells were frequently observed using an inverted light microscope and they were passaged every 2-3 days (when reaching about 90% confluency). For that, confluent cells were collected and centrifuged at 130 *x g* (Centrifuge 5810R, Eppendorf) for 10 min, the supernatant discarded and the pellet resuspended in fresh medium and transferred into a new cell culture flask.

The cell lines were genotyped and routinely monitored for *mycoplasma* contamination by using the Polymerase Chain Reaction, PCR (VenorGeM® Advance Mycoplasma Detection Kit, Minerva). These analyses were performed by the Service platforms of i3S – Instituto de Investigação e Inovação em Saúde da Universidade do Porto (Cell Culture and Genotyping Service).

All procedures involving cell culture were performed under extremely clean and sterile conditions, in biological laminar flow hoods. Aseptic technique was used for handling sterile reagents and materials. Cell number and viability were routinely analyzed with the trypan blue exclusion assay. All experiments were carried out when cells were in exponential growth and having over 90% viability.

2.2. Cellular viability and proliferation assays

2.2.1. Trypan blue exclusion assay

The cell number was frequently counted using a hemocytometer (*Neubauer Chamber)* and the Trypan Blue reagent (Sigma-Aldrich, Co.), in order to maintain the cells

at an appropriate density (around 5 × 10^5 cells/mL) and to ascertain cell viability values (ideally over 90%). Trypan blue penetrates the membrane of disrupted cells, allowing to distinguish between alive and dead cells (blue ones).

2.2.2. Resazurin assay

The resazurin assay is based on the capacity of metabolically active cells to reduce the dye resazurin into resorufin – a pink fluorescent product that can be easily measured by fluorescence, using a 530 nm excitation and 590 nm emission filter set [141, 142]. Thus, resazurin is a cell permeable redox indicator that can be used to monitor viable cells.

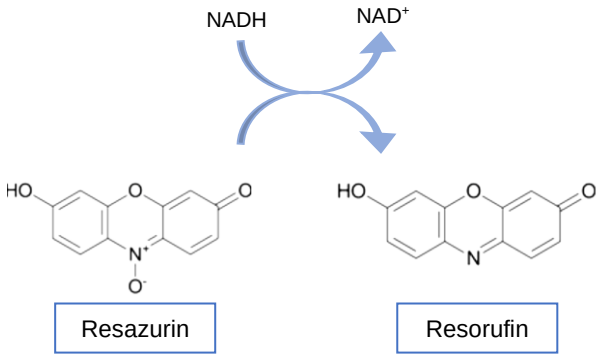


Figure 8. Resazurin reduction reaction from metabolically active cells.

2.2.2.1. Dose-response curves of KBM5 and KBM5-STI cells to imatinib mesylate (STI571)

Dose-response curves to imatinib (STI571) were performed, to confirm the respective sensitive and resistant phenotypes of the KBM5 and KBM5-STI cell lines and to determine the drug's concentration which inhibits cell growth by 50% (IC50).

To that end, 80 µL of cellular suspensions of both cell lines (in the optimal concentration previously determined by other members of the team – 1.5 x 10^5 cells/mL) were seeded in a 96-well plate. After 24 h, 10 µL of a range of different concentrations of STI571 were added to the cells. Ten different concentrations of STI571 were tested: 0.04 µM, 0.08 µM, 0.15 µM, 0.31 µM, 0.63 µM, 1.3 µM, 2.5 µM, 5.0 µM, 10 µM and 20 µM. The higher concentration of STI571 (20 µM) was prepared from a stock of 10 mM (dissolved in DMSO, stored at -20 ºC and protected from light) and the following concentrations were prepared by successive dilutions. These concentrations were selected taking into account

the IC50 values described in the literature [140]. After 48 h treatment with STI571, 10 µL of resazurin solution (1:9 proportion, Sigma-Aldrich, Co.) was added to each well, and the fluorescence was measured following 4 h incubation period at 37 ºC, protected from light. Fluorescence was then measured at both 530 nm and 590 nm (excitation and emission wavelengths, respectively) using a microplate reader (BioTek's Synergy™ Mx) and the Gen5 software (BioTek). The IC50 (the concentration of STI571 that inhibits cell growth in 50%) was then determined by interpolation from the obtained curves. The schematic representation of the plate is shown in **Annex 1**.

Results were presented by the average plus or minus standard errors from at least 3 independent experiments, generated by Excel.

2.3. Extracellular Vesicles (EVs) isolation

EVs were isolated from KBM5 and KBM5-STI cells using the ultracentrifugation method, as previously described [143]. Cells were cultured during 3 days in EV-depleted culture medium (medium containing FBS which had been ultracentrifuged at 100 000 x *g* overnight, at 4ºC and filtered with a 0.2 µM pore filter, in order to remove EVs from the serum). Then, EVs were isolated following a sequential centrifugation procedure, as summarized below **(Figure 9)**.

First, the cell suspension was centrifuged at 200 x *g* (centrifuge 5810R, Eppendorf) during 10 min to remove the cells. The succeeding supernatant centrifugation steps were carried out at 4 ºC in the same centrifuge: a centrifugation during 10 min at 300 x *g* to remove remaining cells and dead cells, followed by a centrifugation during 10 min at 2 000 x *g* to remove cell debris and apoptotic bodies. Next, the supernatant was centrifuged using a High Speed Centrifuge (AVANTI J-25, Beckman Coulter), for 30 min at 10 000 x *g* to remove other large vesicles. Then, the supernatant obtained was ultracentrifuged (Optima XE-100 Ultracentrifuge, Beckman Coulter) during 70 min at 100 000 x *g* in order to pellet the intended EVs (mostly microvesicles and exosomes). The pellet was finally washed in PBS and again ultracentrifuged at 100 000 x *g* for 70 min to remove medium and secreted proteins [144]. The ultracentrifugation steps were carried out using the Type 70 Ti Rotor, Fixed Angle, with the 26.9 ml Quickseal tubes.

In addition, after each centrifugation, the supernatants were carefully transferred into new tubes without disturbing the pellets, in order to avoid contamination. Tubes used in these centrifugations were prior washed with a proper detergent (DERQUIM LM 01 Alkaline

LIQUID, from PVL - Produtos para Laboratório, Lda), avoiding microbiologic contaminations and contaminations among samples and experiments.

Whenever EVs were isolated to further co-culture experiments, all isolation procedures were performed under sterile conditions, using autoclaved tubes (20 min at 121ºC) and performing inside of a laminar flow hood.

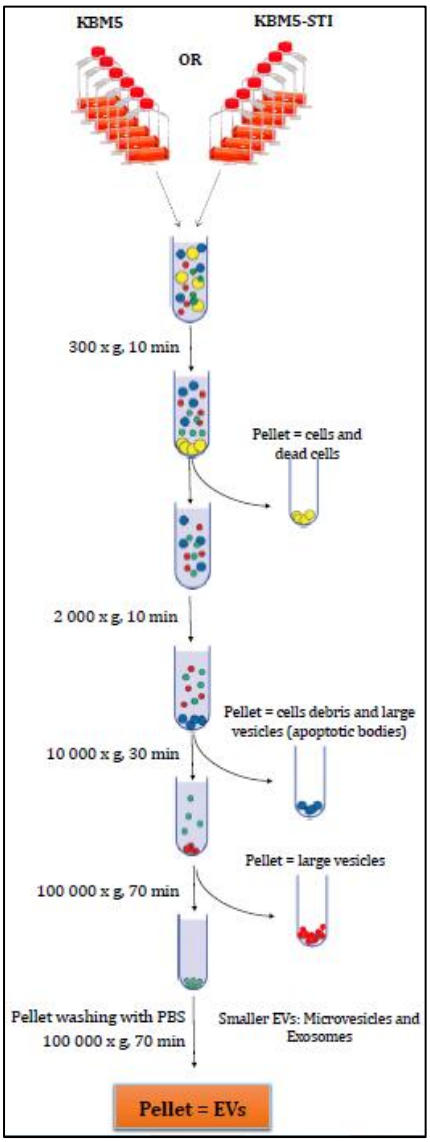


Figure 9. Simplified scheme of the process of EVs isolation by the differential ultracentrifugation method.

2.4. EVs characterization

The EVs isolated by ultracentrifugation were then analyzed and characterized by size and protein content, using the methods described below.

2.4.1. Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) was performed to measure the size/diameter of the isolated EVs. The final pellet obtained by ultracentrifugation, which contain EVs, was dissolved in 70 µL of PBS and added to a polystyrene cuvette with 10 mm path length. DLS analysis was performed using the Zetasizer Nano ZS system and software (Malvern Instruments). The mean hydrodynamic diameter of the EVs was calculated by fitting a Gaussian function to the measured size distribution. Measurements were conducted at 25ºC, operating at 633 nm and recording the back scattered light at a 173º angle. Samples' temperature was allowed to equilibrate for 2 min before each measurement. Data obtained corresponds to a graph reflecting size distribution by number, allowing to infer the subpopulations of EVs which compose the sample.

2.4.2. Nanoparticle Tracking Analysis (NTA)

The Nanoparticle Tracking Analysis (NTA) technique also allows characterizing EVs regarding their size. Thus, the EVs pellet (after ultracentrifugation) was dissolved in 70 µL of PBS and then their protein content was quantified using a modified version of the Lowry assay (Bio-Rad DC Protein Assay), as further described below, in section 4.4.1.. PBS was used as a Blank. Then, for each sample, the volume corresponding to 1 µg of EVs' protein was vigorously vortexed and aspired to a syringe at a constant speed, into a NanoSight NS300 system (Malvern Instruments, U.K.). This equipment makes a fast video capture and its particle-tracking software is able to measure the Brownian movement rate of the particles. All camera settings were fixed and maintained constant for all measurements. EVs's size was inferred by the velocity of the particles (with the principle that smaller particles are faster than bigger particles).

For EVs' size distribution analysis it was preferentially used the mode parameter (although the program gives the mean and mode analysis), in order to exclude possible aggregates that can overestimate the results.

2.4.3. Transmission Electron Microscopy (TEM)

The transmission electron microscope (TEM) allows visualization of EVs and thus confirming their presence in our experiment. Thereby, the EVs pellet obtained by ultracentrifugation was resuspended in approximately 50 µL of PBS. Then, 10 to 15 µL of this solution were added into Formvar-carbon coated electron microscopy grids for 1-2 min in the dark at RT (for coating). Samples were allowed to dry and 5 µL of uranyl acetate (contrasting solution) was added and the preparation was observed under a transmission electron microscope (TEM, Jeol JEM 1400) at an acceleration voltage of 80 kV. Data was obtained by the Histology and Electron Microscopy Service of IBMC/i3S, Porto.

2.4.4. Detection of EVs markers (Western Blot)

2.4.4.1. Protein extraction and quantification

The EVs pellet obtained by ultracentrifugation was lysed in 30 µL of lysis Winman's buffer (1% NP-40, 0.1 M Tris-HCl pH 8.0, 0.15 M NaCl and 5 mM EDTA) complemented with EDTA-free protease and phosphatase inhibitor cocktail (Roche), for 30 min at 4 ºC (with agitation). After centrifugation at 16 200 x *g*, during 10 min at 4ºC (Micro Star 17R, VWR), the supernatant (corresponding to the proteins) was collected and protein was quantified using a method based on the Lowry protocol (Bio-Rad DC™ Protein assay). The appropriate standards of bovine serum albumin (BSA, from Sigma-Aldrich, Co.) were used to generate a standard curve and ultrapure H_2O was used as Blank. Absorbance was measured in a microplate reader at 655 nm after 15-30 min incubation in the dark.

2.4.4.2. Protein expression analysis by Western Blot (WB)

After protein quantification, 5 to 10 µg of EVs' protein lysates were mixed with Loading Buffer (Tris-HCl 1 M, 5% SDS, 12% Glicerol, 12% β-mercaptoethanol and 0.024% bromophenol blue), boiled for 5 min at 95ºC (for protein denaturation) and separated on 8%, 10% or 12% Tris-glycine SDS-Page polyacrylamide gels at 70 V (for at least 30 min) followed by approximately 1 h and 30 min at 100 V. The running buffer used was prepared from a commercial 10x Tris/Glycine/SDS buffer solution (Bio-Rad). After the separation, proteins were transferred onto a nitrocellulose membrane (Amersham Protran 0.45 NC, GE Healthcare) for approximately 2 h at 100 V. Transfer buffer was prepared from the commercial 10x Tris/Glycine buffer solution (Bio-Rad) to which 20% of methanol was

added. All the procedure was done in a Bio-Rad Western Blot System. Before proceeding, membranes were stained with Ponceau S solution (Sigma-Aldrich, Co.) to guarantee that all wells were correctly loaded with the same amount of protein and to confirm the successful transference of the protein onto the membranes.

Membranes were then blocked in TBS-T [a Tris-buffered saline solution with 0.1 % Tween-20 (Promega)], containing 5% (w/v) non-fat dry milk (Molico), for at least 30 min with agitation at room temperature (RT). Primary and secondary antibodies were prepared using this solution. Membranes were incubated with the primary antibodies for 1 h and 30 min at RT or overnight at 4ºC, washed 3 times in TBS-T during 10 min with agitation and then incubated with the corresponding secondary antibodies for 1 h at RT. The antibodies used and their corresponding dilutions were: anti-Syntenin-1 (S-31): sc-100336 1:200; anti-CHMP4B (C-12): sc-82556 1:100; anti-Clathrin LCB (H-60): sc-28277 1:200; anti-Flotillin-1 (F-3): sc-74567 1:1000 and anti-cytochrome *c* (7H8); sc-13560 1:1000, from Santa Cruz Biotechnology; anti-CD63 (EXOAB-CD63A-1) 1:1000, and anti-Hsp70 (EXOAB-Hsp70A-1) 1:500, from System Biosciences. The loading controls used were anti-tubulin (T6074) 1:10 000, from Sigma-Aldrich, Co. or anti-Actin (sc-1616) 1:2000, from Santa Cruz Biotechnology. The following secondary antibodies were used: goat anti-rabbit IgG-HRP (sc-2004); goat anti-mouse IgG-HRP (sc-2031); and donkey anti-goat IgG-HRP (sc-2020), from Santa Cruz Biotechnology, all of them at a dilution of 1:2000. After washing the membranes 3 times in TBS-T for 10 min, the signal from the membranes was detected using the ECL Western Blot Detection Reagents (GE Healthcare), the chemiluminescence Amersham Hyperfilm ECL (GE Healthcare) and the Kodak GBX developer and fixer (Sigma-Aldrich, Co.). The intensity of the bands was analyzed using the software Quantity One – ID Analysis (Bio-Rad).

Whenever necessary, stripping of the membranes was done. The stripping buffer used contained 10% methanol and 10% acetic acid (Sigma-Aldrich, Co.). Membranes were washed 15 min with TBS-T prior and after stripping.

2.5. Analysis of BCR-ABL levels in cellular and EVs' extracts

2.5.1. BCR-ABL oncoprotein analysis by Western Blot

Protein extraction, quantification and analysis were performed as described in the previous section. For the preparation of cellular extracts, approximately 1 mL of cellular suspension of KBM5 and KBM5-STI cell lines was centrifuged at 130 x *g* for 5 min, washed

with 1 mL PBS and centrifuged again at 200 x *g* for 5 min at 4 ºC. After removing the PBS, pellets were lysed in approximately 30 µL of lysis buffer. Then, 5 to 50 µg of cells' protein lysates and 5 to 10 µg of EVs' protein lysates were separated in a polyacrylamide gel and further transferred to a membrane.

The following commercial primary antibodies were used: anti-c-Abl (24-11): sc-23 1:100 and p-c-Abl (7.Tyr 412): sc-293130 1:100 (in this case, block was done in TBS-T containing 5% (w/v) of BSA) from Santa Cruz Biotechnology. The loading controls and the secondary antibodies were the same as mentioned before.

2.5.2. *BCR-ABL* mRNA

2.5.2.1 RNA extraction

Suspension cells (5-10 x 10^6 cells) were pelleted by 800 x g centrifugation, 10 min, and the supernatant discarded. Then, it was added 1.5 mL Trizol ™ Reagent (Ambion) and the pellet was vigorously homogenized, on ice, in order to completely disrupt the cells. After incubation of the homogenate for 5 min at RT, 200 µL of chloroform (Sigma-Aldrich, Co.) per 1 mL Trizol was added and the mixture was stirred and vortexed vigorously. The mixture remained 2-3 min incubating at RT, to allow the total dissociation of the nucleoprotein complexes. After the centrifugation at 12 000 x *g* for 15 min at 4°C, a separation of phases occurred: an upper aqueous phase containing RNA; a white interphase with DNA and proteins; and a lower red phase corresponding to organic reagents. The colorless top layer was transferred to a fresh tube, and 500 µL of isopropanol (Sigma-Aldrich, Co.) per 1 mL of Trizol were added for lysis (RNA precipitation). Samples were incubated in this solution overnight at 4ºC and then centrifuged for 10 min at 12 000 x *g* at 4ºC. After removal of the supernatant, precipitated RNA was washed in approximately 1 mL of cold 75% ethanol (Fisher Chemical) per 1 mL of Trizol initially added and centrifuged at 7 500 x *g* at 4ºC for 5 min. Ethanol was removed and the pellet was left air drying until it got colorless, when it was immediately resuspended in 10-25 µL ultrapure water (depending on pellet size).

RNA yield and quantification were assessed by spectrophotometry at 260 and 280 nm, using NanoDrop™ 1000 Spectophotometer (Thermo Scientific). A 1% agarose (SeaKem® LE Agarose, Lonza) gel electrophoresis, stained with GreenSafe Premium (NZYTech, 3 µL for each 100 mL of solution), was performed to verify RNA quality (absence of DNA contamination and RNA degradation). TBE buffer (Tris/Borate/EDTA, from Grisp)

was used to prepare the gel and also used as electrophoresis buffer. Loading buffer Orange G (Sigma-Aldrich, Co.) was added to each sample and the running was performed at 100 V during approximately 30 min. The gel was visualized by a gel transilluminator, Geldoc™ XR+, using the Image Lab software (BioRad).

The same procedure was performed to RNA extraction from EVs, but some additional steps were carried out prior it in order to remove extra RNA that might be outside of the EVs (and so, the analysis be only from the RNA inside of EVs) [145].

For that, proteinase K (0.05 µg/µL, from Sigma-Aldrich, Co.) was added to the isolated EVs and incubated for 10 min at 37ºC. Then, to inhibit its activity, 5 mM of phenylmethylsulfonyl fluoride (PMSF, from Sigma-Aldrich, Co.) were added for 10 min at RT. In order to further inactivate proteinase K activity, the mixture was incubated at 90ºC for 5 min. Then, RNAse A (0.5 µg/µL, from Thermo Fischer Scientific) was added to the samples and incubated for 20 min at 37ºC, in order to unprotected RNA be degraded. Ultimately, RNA was extracted as described formerly.

All the purified RNA samples were stored at -80ºC. During extraction, every step was performed at a cleaned workplace to avoid RNAse contamination, inside a hotte prior cleaned with ethanol 70% and/or with RNAse away solution (Sigma-Aldrich, Co.). Filtered tips were used.

2.5.2.2. *BCR-ABL* quantification

To assess the presence and copy numbers of *BCR-ABL* in cells and EVs, complementary DNA (cDNA) synthesis by reverse transcription and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) were performed from 1 µg of RNA of each sample. This procedure was carrying out by our team collaborators from the Department of Hematology of Hospital São João (Oporto). In addition, *ABL1* was used as reference gene and the percentage of *BCR-ABL* was obtained in relation to it.

2.6. Co-culture of sensitive cells with EVs released by their drug resistant counterparts

Isolated EVs obtained by ultracentrifugation were resuspended in 100 µL of PBS, quantified using the modified Lowry protocol (as mentioned above in **section 2.4.4.1.**) and frozen at −80°C.

In order to verify if the KBM5 cells (sensitive cells) gain resistance to STI571 following incubation with EVs released by the resistant cells (KBM5-STI), co-culture experiments were performed. For that, co-culture was done in 3 different 96-well plates, with 80 µL per well of KBM5 and KBM5-STI cells seeded at a cellular density of 1.5 x 10^5 cells/mL and 8.0 x 10^5 cells/mL, depended on the experiments. After approximately 12 h and 24 h, cells suffered different treatments: both resistant and sensitive cells were treated with PBS (as a control), KBM5 cells were treated with EVs released by themselves (as a control) and KBM5 cells were treated with EVs released by their resistant counterpart cells (KBM5-STI) during different times (6 h, 24 h or 48 h). An additional plate was used as a control of cell growth (named T0 plate), in order to have information on cells proliferation from the beginning to the end of the experiment. Therefore, the cell growth in the T0 plate was read 24 h after plating, by addition of resazurin. The amount of EVs used in the same experiment was equal, but different amounts were tested (5 µg, 10 µg and 15 µg) in different experiments. At the end of the above indicated time points (6 h, 24 h or 48 h), all conditions were incubated with STI571 for further 24 h or 48 h. In the end, the percentage of metabolically active cells was assessed with the resazurin assay, as describe above **in section 2.2.2.1.. Annex 2** outlines the general schematic representation of the plate used for these experiments.

In addition, co-culture experiments were performed using RPMI medium with 10% of depleted FBS (vesicles-free serum) and 1% of Antibiotic Antimycotic Stablized Solution (Sigma-Aldrich, Co.).

2.6.1. Optimization of KBM5 and KBM5-STI cells concentrations to be plated

The determination of the ideal cell concentration to be plated in the 96-well plates for KBM5 and KBM5-STI cell lines was performed. Thus, different cell concentrations were tested: 2.5 x 10^4, 5 x 10^4, 8 x 10^4, 1.5 x 10^5, 3 x 10^5 and 6 x 10^5 cells/mL. This experiment was performed for three different time-points, as explained above. The following protocol was used: in 3 different 96-well plates (for the 3 different time-points) 80 µL of different cell concentrations of KBM5 and KBM5-STI cell lines were plated per well (prepared by successive dilutions from the higher concentration). The Blank treatment consisted of 80 µL of culture medium only. Cells were then incubated during 24 h, 48 h or 72 h, and after that

10 µL of medium was added per well (corresponding to the volume of compounds that will be added in further experiments). Cells were incubated for another 48 h. Then, half of the volume from each well was collected for cell counting by trypan blue exclusion assay and the other half was used in the resazurin assay. For that, resazurin solution at 0.1 mg/mL was added to each well at 1:9 proportion. The plates were incubated and read as mentioned in **section 2.2.2.1..** The schematic representation of the plate is shown in **Annex 3.**

2.7. Statistical analysis

Data were summarized by means and standard errors of the means for symmetric distributions in continued variables. For data normally distributed, the statistical analyses were performed using paired and unpaired two-tailed Student's t-test. To consider a statistical significant data, a *p* value <0.05 was established.

III. Results and Discussion

3.1. Characterization of the counterpart pair of cell lines

The cell lines used in this study were a pair of drug-sensitive (KBM5) and drug-resistant (KBM5-STI571, or to simplify, from now on, KBM5-STI) counterparts. The resistant cell line was derived from the sensitive (parental) one by treatment with increasing concentrations of imatinib mesylate (STI571) during several months, until the selected cell line presented normal growth kinetics in the presence of 1 μM of the drug [70, 139, 140]. As previously referred, both cell lines were kindly provided to our group by the laboratory that established the resistant cells.

KBM5 and KBM5-STI are Philadelphia chromosome positive cell lines. KBM5 cells were established from a 67-year-old female CML patient in the blastic phase of the disease. Treatment of the cells with low concentrations of STI571 for a long period of time allowed those scientists to select the cells that survived in presence of STI571. It was verified that the selected resistant cells had a T315I single point mutation in BCR-ABL, causing a substitution of a threonine by an isoleucine at the Thr-315 residue of ABL [140, 146].

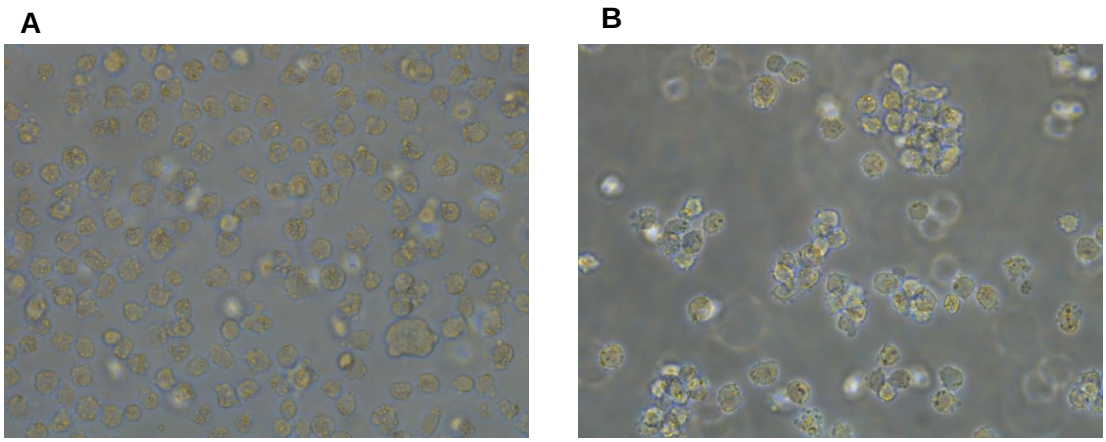


Figure 10. Microscopic images (200X) of the KBM5 (A) and KBM5-STI (B) cell lines. Images were obtained using an inverted light microscope (Leica DMi1); both cell lines had been similar time in culture when the pictures were taken.

In order to further study both cell lines, we started to confirm the IC50 described in the literature, using the resazurin assay. The ideal cell density to be plated had been previously optimized in our laboratory. Thus, 1.5 x 10^5 cells/mL of KBM5 and KBM5-STI cell lines (corresponding to the exponential phase of cell growth without reaching confluence during the period of the experiment) were seeded in each well and, after 48 h of drug treatment with different concentrations of STI571, the IC50's were determined for each cell line. For each of the tested concentrations of the drug, the percentage of cells metabolically

active was calculated by normalization of the values obtained for drug-treatments with those obtained in cells growing in normal medium, without the presence of the drug. Moreover, the presence of DMSO in a volume equivalent to the highest drug concentration tested proved to be non-toxic.

Results presented in **Figure 11** and **Table 1** confirmed the two distinct profiles of the counterpart pair of cell lines in response to treatment with STI571: KBM5 cells were sensitive to STI571 while the KBM5-STI cells were more resistant to STI571 treatment up to the highest concentrations of the drug used (20 µM – concentration to which both cell lines responded similarly). The concentration of STI571 that inhibited the metabolic activity of KBM5 cells by 50% was 0.11 µM, while for KBM5-STI cells was 11.57 µM. These results are similar to those published in the literature for these cell lines [140].

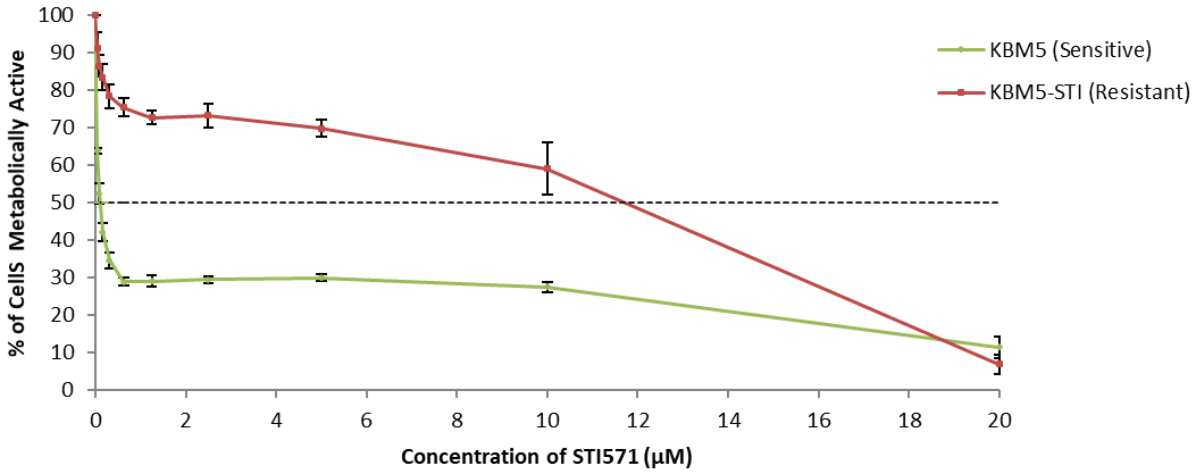


Figure 11. Dose-response curves of KBM5 cells (green line) and KBM5-STI cells (red line) to imatinib mesylate (STI571). Cells were treated with different concentrations of STI571 and the percentage of cells metabolically active was assessed after 48 h of drug treatment, using resazurin assay. Results are the mean ± S.E. of 4 independent experiments.

Table 1. The concentration of imatinib mesylate (STI571) that inhibits KBM5 and KBM5-STI cellular viability by 50% (IC50). These values were obtained by the interpolation from the obtained dose-response curves (i.e. interpolation of the straight line corresponding to YY=50 with each curve).

Cell line	IC50 ± S.E. (µM)
KBM5	0.11 ± 0.01
KBM5-STI	11.57 ± 1.30

3.2. Characterization of Extracellular Vesicles (EVs) derived from the counterpart pair of cell lines

The EVs research area is relatively new, however several protocols for their extraction have been developed and optimized. Some of those protocols are based on the following: size exclusion chromatography, which uses columns with a stationary and mobile phase; immunoaffinity isolation, with microbeads; polymeric precipitation with commercial kits, e.g. ExoQuick^{TM}; and ultracentrifugation, consisting of successive centrifugations until obtaining an EVs pellet [143] [147]. In this work, EVs derived from both cell lines were isolated by ultracentrifugation, the most commonly used method and the gold-standard technique for EVs isolation so far. The ultracentrifugation protocol is based on the particles' size and consists of various differential centrifugation steps [148].

Once the EVs are isolated, it is important to characterize them, not only to confirm the isolation of EVs but also to study their characteristics. **Figures 12 to 15** show results from several analyses used for EVs characterization. EVs were characterized in terms of size (**Figures 12 and 13 and Table 2**) by DLS and NTA, in terms of morphology (**Figure 14**) by TEM and in terms of protein content (**Figure 15**) by Western blot.

Regarding the DLS results (**Figure 12**), it is possible to verify a particle size distribution from 10 to 1000 nm, similar for the EVs isolated from both cell lines. This range of sizes encompasses the sizes described for EVs (in which exosomes are about 30 to 100 nm and microvesicles are about 50 to 1000 nm) [144]. In addition, we verified that the smaller vesicles (with less than or around 100 nm) were the main subpopulation of EVs extracted from both cell lines. Results from the DLS analysis were plotted as particle number vs size, instead of particle intensity vs size (the direct measurement of the Zetasizer equipment) since bigger particles diffract more light, which will be reflected in a higher peak in the graph of intensity, but that does not mean that this subpopulation of EVs is more abundant in the sample.

Therefore, we used this representation of results (as particle number vs size) to avoid an incorrect interpretation.

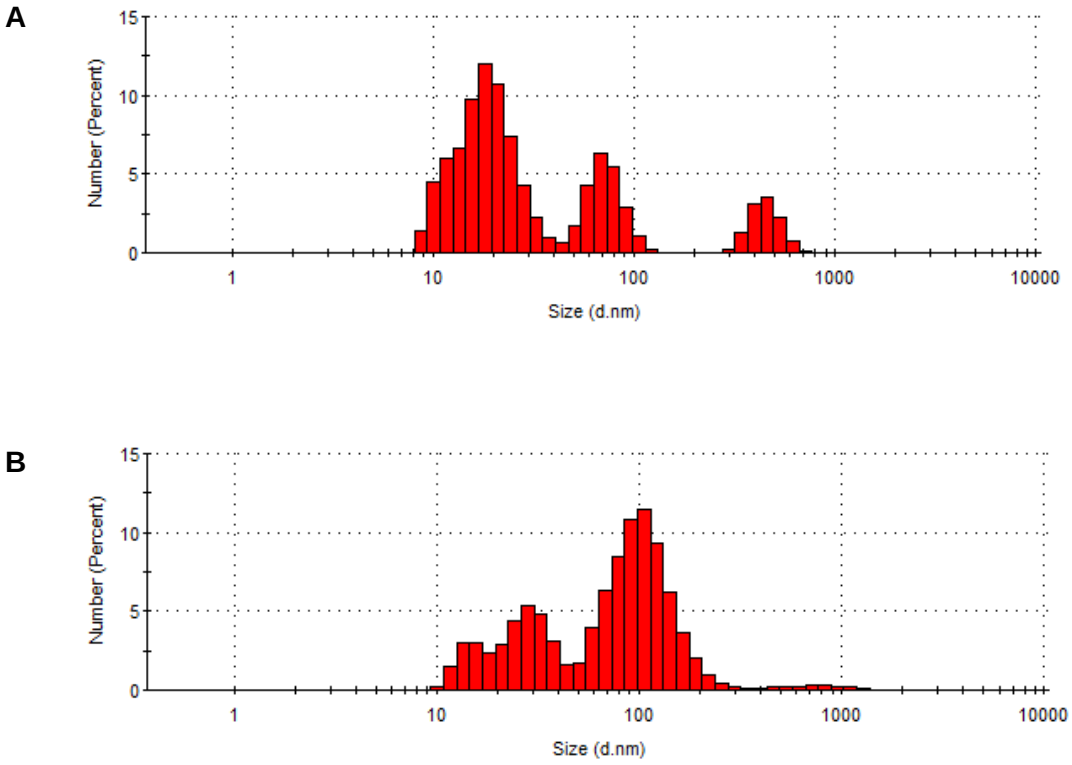


Figure 12. Size distribution of EVs isolated from KBM5 (A) and KBM5-STI (B) cell lines, analyzed by DLS. Results are the mean of 3 independent experiments, analyzed with the Zetasizer software.

According to NTA results, isolated EVs have a size ranging from 10 to 500 nm, with a prevalence of EVs with sizes of approximately 100 nm (**Figure 13 and Table 2**). Therefore, both NTA and DLS analysis indicate that the EVs extracted have the same prevalence of smaller vesicles (equal to or less than 100 nm). Of note, the NTA provides not only information regarding the EVs' size distribution but also on EVs concentration (particles/mL). Results regarding the concentration of particles showed that there were no significant differences between the size distribution of the EVs isolated from the two counterpart cell lines ($p>0.05$, obtained from two-tailed unpaired Student's *t*-test) (**Table 2**). These results are in accordance with the ones obtained by the Lowry-based method (which allowed quantifying the total protein content of EVs) previously used to quantify 1 µg of protein and inject the corresponding volume in the equipment for NTA analysis. Since the same amount of protein was injected for all samples and the same amount of particles per mL were obtained in the NTA analysis, we concluded that both methods are useful to quantify EVs.

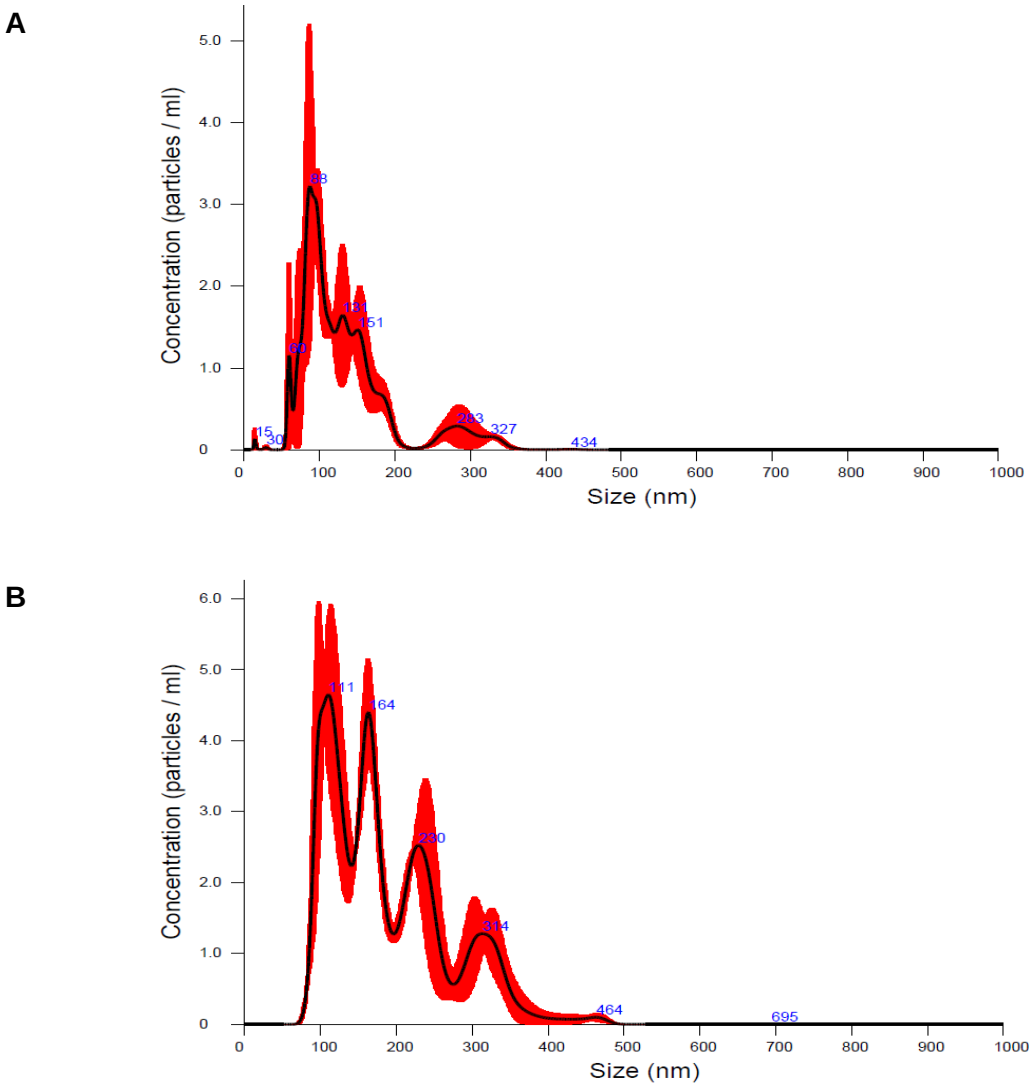


Figure 13. Size distribution of EVs isolated from KBM5 (A) and KBM5-STI (B) cells, analyzed by NTA. Each graph is representative of 3 independent experiments, generated by Nanosight software.

Table 2. Summary of the parameters measured by NTA of EVs isolated from KBM5 and KBM5-STI cell lines. Results are the mean of 3 independent experiments, generated by Nanosight software.

Cell line-derived EVs	Mean ± S.E. (nm)	Mode ± S.E. (nm)	Concentration ± S.E. (particles/mL)
KBM5	154.8 ± 6.4	105.0 ± 9.0	$3.36 \times 10^8 \pm 4.75 \times 10^7$
KBM5-STI	193.2 ± 4.2	129.2 ± 8.2	$4.58 \times 10^8 \pm 5.55 \times 10^7$

Another technique used to characterize EVs is TEM. This methodology allows the visualization of the structure, shape and size of EVs. Thereby, the presence of EVs following

the isolation protocol was further confirmed by TEM. In **Figure 14**, it is possible to observe the cup shape vesicles of different sizes, by negative staining. Thus, it was possible confirm that EVs from KBM5 and KBM5-STI cells were successfully isolated using the ultracentrifugation methodology. In addition, we verified that EVs released by KBM5 and KBM5-STI cells are mainly composed of small-size EVs.

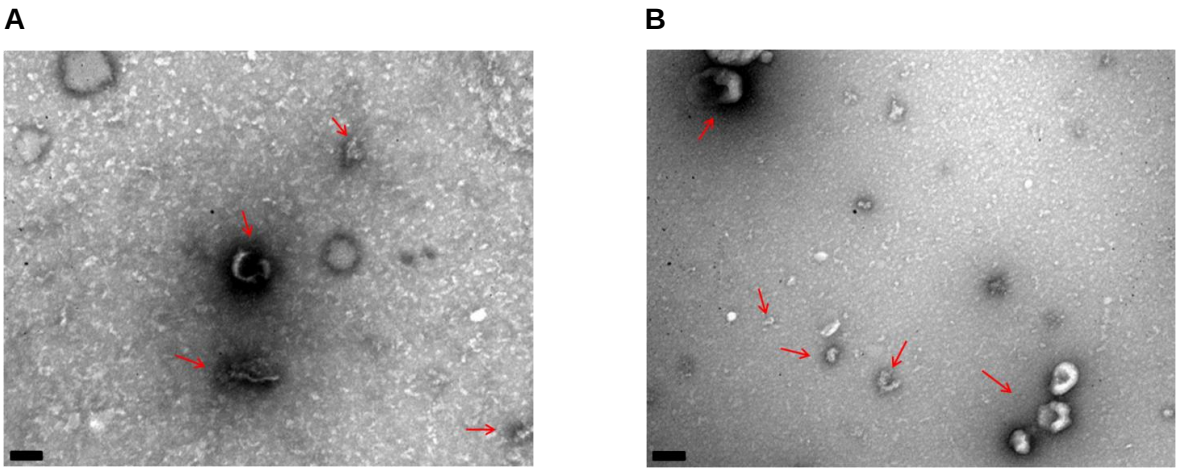


Figure 14. Morphology of EVs isolated from KBM5 (A) and KBM5-STI (B) cells, analyzed by TEM. Images are representative of 2 independent experiments. Red arrows indicate the EVs detected with this methodology. Scale bar = 100 nm.

Next, in order to characterize those EVs released by KBM5 and KBM5-STI cells, we investigated the presence of EV markers by Western blot analysis. Some classical markers, mostly involved in EVs biogenesis, were analyzed. Particularly, the markers studied were membrane-bound proteins, namely the tetraspanin CD63, CHMP4B (the component of the endosomal sorting complex required for transport, ESCRT III) and cytosolic proteins such as the heat-shock protein HSP70, syntenin-1 and flotillin-1 [109, 143, 144].

The cytochrome *c* (cyt *c*), a cell organelle marker, was assessed in order to exclude possible cellular debris contamination in the population of isolated EVs and thus was used as a negative control.

Cytoskeletal proteins, like actin and tubulin, are frequently used as loading controls for cellular extracts. Nevertheless, it is not easy to find a good protein that may act as *housekeeping* in the case of EVs, since their cargo varies depending on the donor cell and is not an exact representation of the donor cell components. For that reason, some authors do not include loading controls for EVs protein lysates and, in some cases, they show the Ponceau staining instead, as prove of the amount of protein loaded into each well [149].

In fact, Western blot is a challenging procedure in the EVs field, since it is necessary to start from a substantial amount of biological material (high quantity of cells from several culture flasks) in order to obtain an achievable amount of EVs extracts and enough protein to load a gel.

As shown in **Figure 15**, we observed the presence of all the studied EVs markers in the EVs released from KBM5 and KBM5-STI cells. In addition, it was possible to confirm the absence of cellular contaminants, by the lack of detection of cyt *c* in the EVs extracts. Thus, we confirmed the presence of EVs in our extracts isolated by ultracentrifugation.

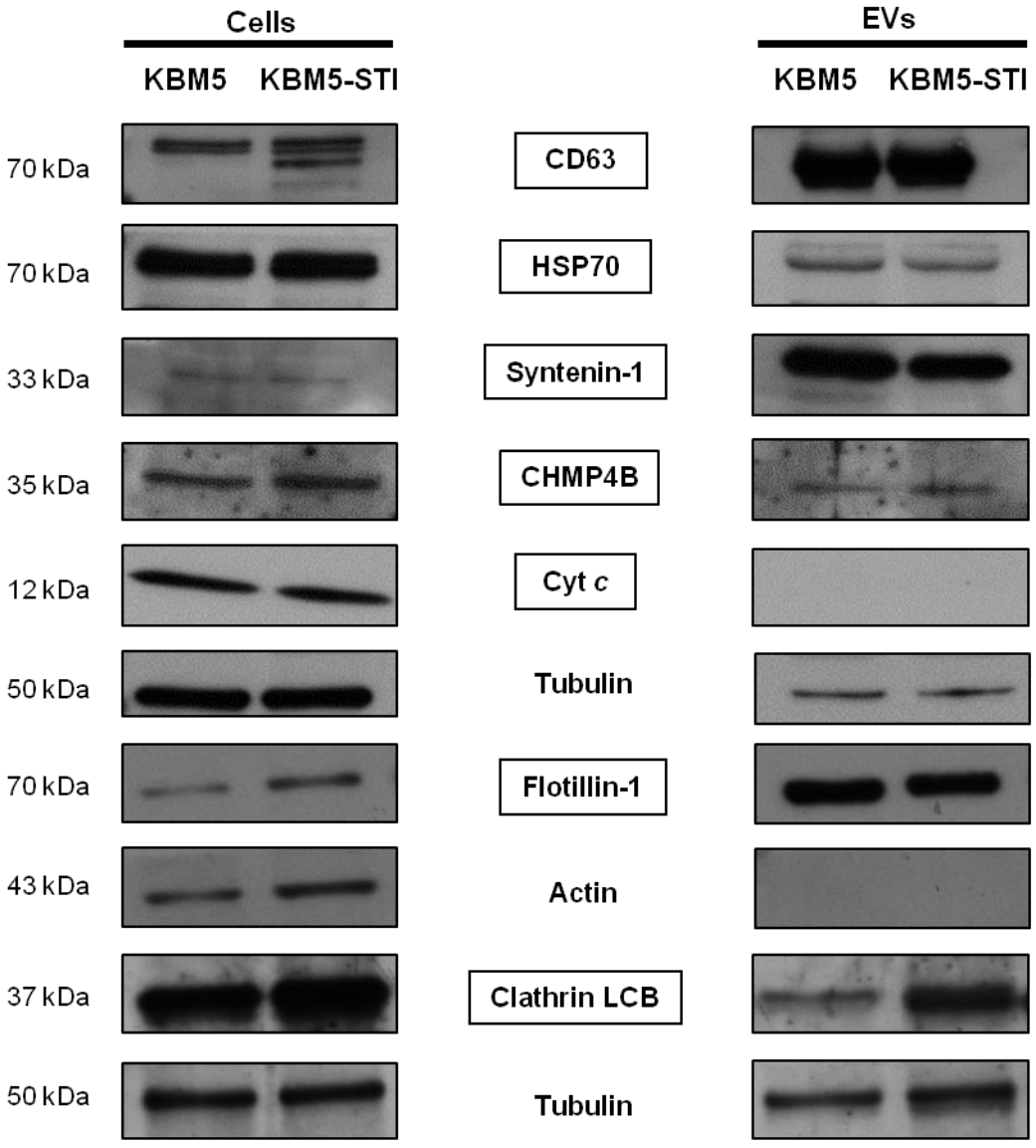


Figure 15. Analysis of several classical EVs markers in KBM5 and KBM5-STI cells and in the EVs released by those cells, by Western blot. Cells and EVs have the same amount of protein loaded in each case. Blots are representative of 1 (clathrin LCB), 2 (CHMP4B and cyt *c*), 3 (flotilin-1), 4 (syntenin-1) or 5 (CD63 and Hsp-70) independent experiments. Cytochrome *c* was used as control for cellular content. Tubulin and actin were used as loading controls.

Taking together this part of the work, the results obtained here allowed to assure that EVs were successfully isolated by ultracentrifugation, given the size-ranges of the isolated particles (10-1000 nm), the presence of EVs markers and the absence of cellular contaminants. Furthermore, the EVs released from both KBM5 and KBM5-STI cell lines presented similar sizes (mainly small EVs) and protein markers.

To the best of our knowledge, the characterization of EVs isolated from KBM5 and KBM5-STI cell lines has never been described before.

It is important to note that the EVs research field has grown immensely in the past decade, together with its challenges and associated complexity. The need for a transparent and unambiguous interpretation and presentation of results relating to EVs was already noticed and the scientific community has been making an effort for standardization of the most possible parameters, from practical issues (such methods of EVs isolation and analysis), to the treatment of the results, in order to facilitate their comparison and repetition. However, the implementation of such practices is still an ongoing international effort [150-153].

3.3. Investigation of BCR-ABL levels in the counterpart pair of cell lines and in the EVs released by those cells

As BCR-ABL is a key protein of the tumorigenesis of Chronic Myeloid Leukemia (CML), we investigated if cells were exporting this protein by packaging it into the cargo of their EVs. Thus, BCR-ABL protein content was analyzed in KBM5 and KBM5-STI cells and in the EVs released by those cells (**Figure 16A**). In addition, the whole cell lysate from K562 cells (a Philadelphia chromosome positive cell line derived from a CML patient in blastic phase) was used as positive control. The MCF-7 cell line was used as negative control, since this breast cancer derived cell line does not express BCR-ABL. The presence of EVs markers (CD63 and HSP70) was also studied. Ponceau staining was used to show the correct loading of the gel and subsequent transfer to the membrane (**Figure 16B**).

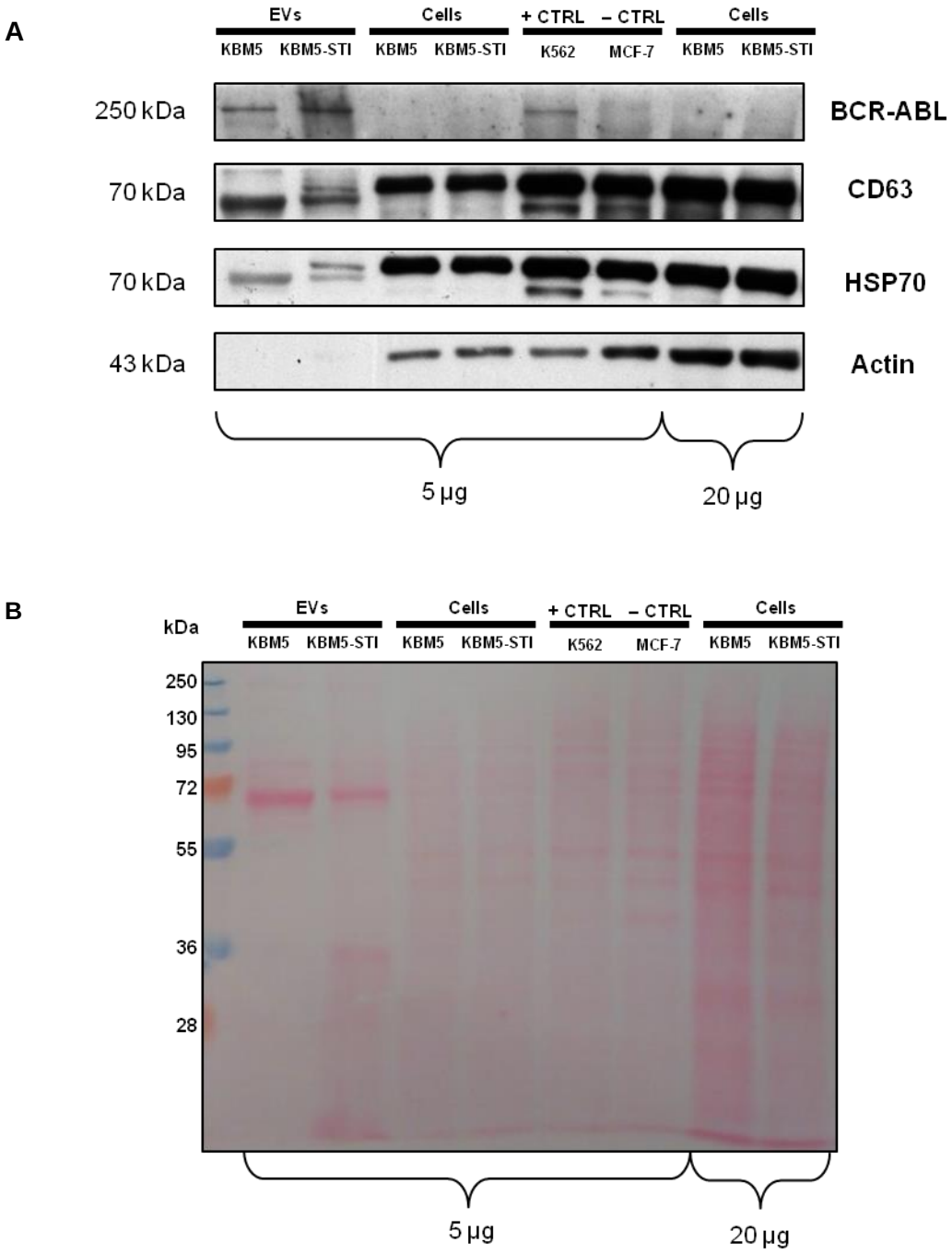


Figure 16. Analysis of BCR-ABL protein content in EVs and cells, by Western blot (A) and corresponding Ponceau protein staining (B). In the first six lanes, 5 µg of protein were loaded. In the last two lanes 20 µg of protein were loaded. The extracts from K562 and MCF-7 cell lines were used as positive and negative controls for BCR-ABL detection, respectively. CD63 and HSP70 are two classical EVs markers. Actin was used as loading control. The antibody used to recognize the BCR-ABL protein was c-Abl (24-11): sc-23, from Santa Cruz Biotechnology.

Results (**Figure 16**) showed that, for the same amount of protein loaded (5 µg), the BCR-ABL protein was detected in EVs shed by KBM5 and KBM5-STI cells but not in the

cellular extracts of those cells, when using the antibody c-Abl (24-11): sc-23, from Santa Cruz Biotechnology. In addition, even when increasing the amount of protein 4 times (to 20 µg), the BCR-ABL protein was not detected in those cells with this antibody. Thus, it seems that the BCR-ABL protein is being selectively packaged into the EVs shed by these cells. This is an interesting mechanism of oncoprotein shedding, previously described for other oncoproteins such as KIT [154], EGFR [155], KRAS and BRAF [156, 157] (the last two in their mutant form), with impact in pathways that lead to tumor progression. On the other hand, the absence of BCR-ABL detection in the cells when only 5 µg of protein were analyzed could be explained by the small amount of total cellular protein loaded. Indeed, it seems that BCR-ABL might be selectively packaged into EVs and therefore enriched in those vesicles when compared to the total protein content of the EVs.

Indeed, with this antibody, BCR-ABL was only detected in cells when much larger amounts of protein were loaded into the Western blot (30 µg or 50 µg of cellular extract, **Figure 17**). In addition, the normal tyrosine kinase protein c-Abl, which is present in all cells, was also detected in this case.

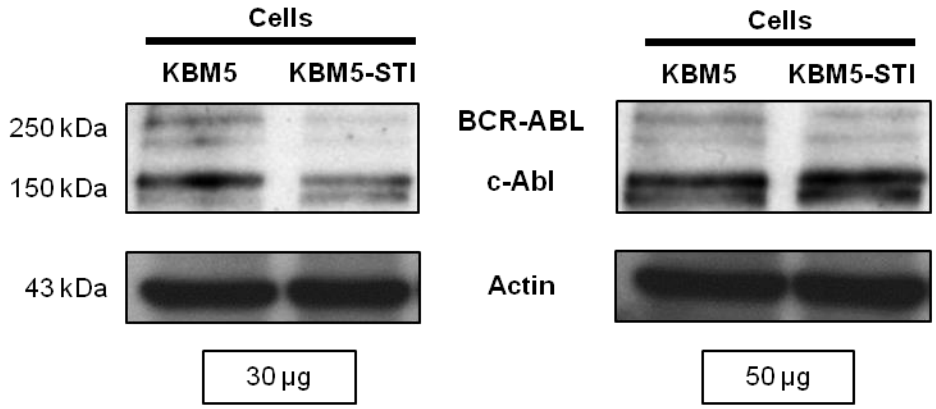


Figure 17. Analysis of BCR-ABL and c-Abl protein content in KBM5 and KBM5-STI cells, by Western blot. A total of 30 µg (left) or 50 µg (right) of cellular protein from KBM5 and KBM5-STI cells were loaded. Actin was used as loading control. The antibody to recognize the BCR-ABL protein used was c-Abl (24-11): sc-23, from Santa Cruz Biotechnology.

The detection of BCR-ABL protein was further confirmed by using another BCR-ABL antibody, the p-c-Abl (7.Tyr 412): sc-293130, from Santa Cruz Biotechnology. Although very faint, a band was detected in both EVs from KBM5 and KBM5-STI cells, confirming the presence of the oncoprotein in these nanoparticles, as shown in **Figure 18**. Again, the positive and negative controls for BCR-ABL were K562 and MCF-7 cells, respectively.

Flotillin-1 is an EV marker, whose detection proved the presence of EVs, and actin was used as loading control, which showed to be only effective in the case of cellular extracts.

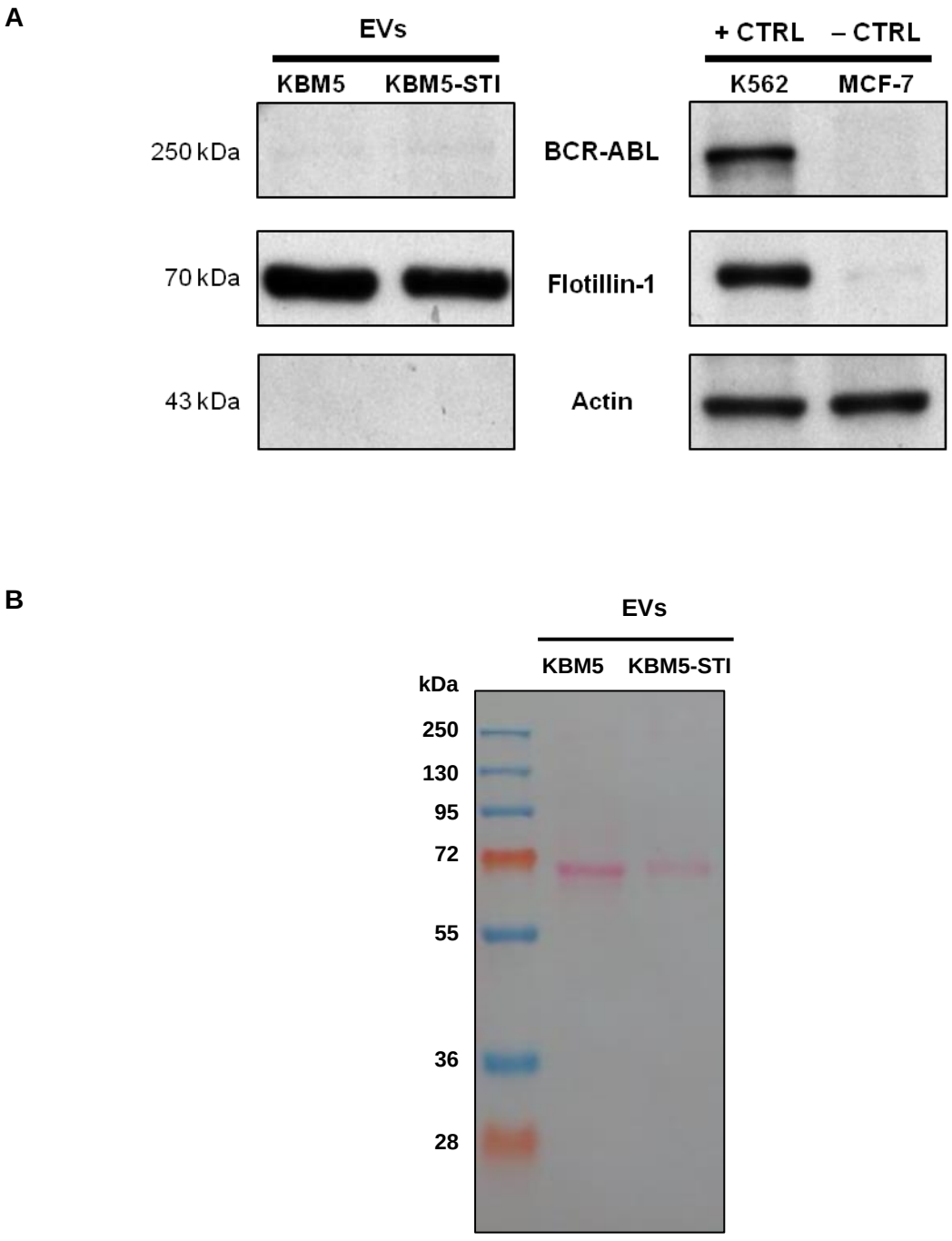


Figure 18. Analysis of BCR-ABL protein content in EVs, by Western blot (A) and respective Ponceau protein staining (B) of the lanes corresponding to EVs. 5 µg of EVs and cells were loaded. The cellular extracts of K562 and MCF-7 were used as positive and negative controls for BCR-ABL detection, respectively. Flotillin-1 is a classical EVs marker. Actin was used as loading control. The antibody to recognize the BCR-ABL protein used was p-c-Abl (7.Tyr 412): sc-293130, from Santa Cruz Biotechnology.

Taken together, these results confirm that the BCR-ABL protein is selectively packaged into EVs which are released from these drug sensitive and drug resistant cells.

Following, it was decided to investigate if the *BCR-ABL* mRNA was also packaged into the EVs. Thereby, RNA from cells and EVs was extracted and analyzed by qRT-PCR for *BCR-ABL1*. *ABL1* was used as reference gene. This work was performed by a collaborator from Centro Hospitalar São João - Porto.

Preliminary results (from one experiment only) suggest that *BCR-ABL* mRNA was also present in EVs released by both cell lines, in a proportion (relative to the reference gene – *ABL1*) similar to the one found in cells (**Table 3**). These results were confirmed by nested PCR (results not shown).

Table 3. Copy numbers of *BCR-ABL1* and *ABL1* transcripts detected in cells and EVs. Preliminary results were obtained by qRT-PCR and are from 1 experiment only.

	Copy number of *BCR-ABL1* transcript	Copy number of *ABL1* transcript
KBM5 Cells	298603.44	416970.25
KBM5-STI Cells	343242.19	463391.59
KBM5 EVs	43.3	63.14
KBM5-STI EVs	584.72	874.48

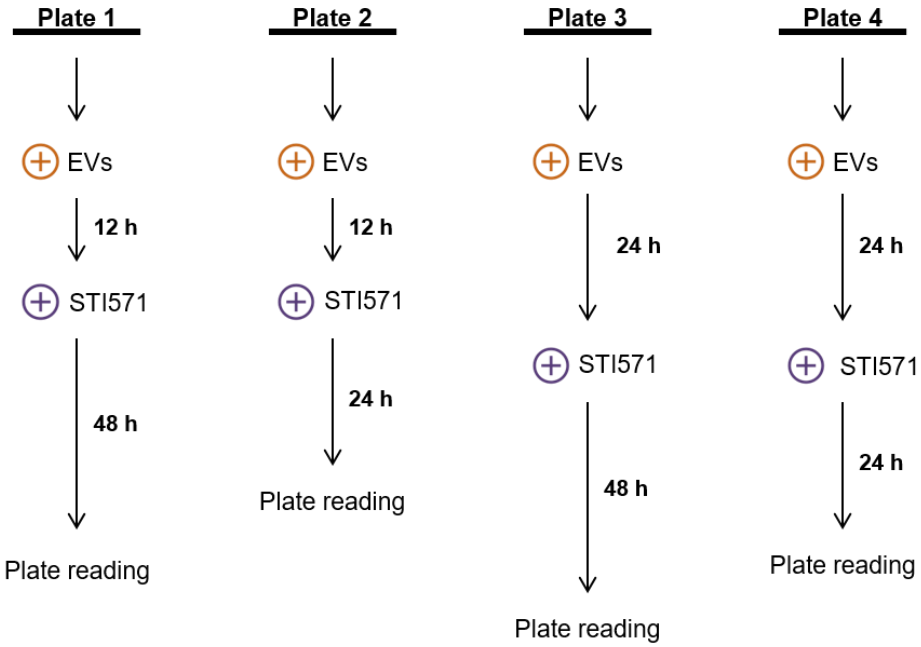
The packaging and release of EVs by K562 cells, containing BCR-ABL gene, transcript and protein was previously demonstrated by other authors, both *in vitro* and *in vivo* [158, 159].

3.4. Effect of co-culture of sensitive cells with EVs released by their drug resistant counterparts

In order to verify if drug-resistant cells transfer resistance to drug sensitive cells, mediated by EVs, co-culture experiments were performed with EVs from drug resistant donor cells with drug sensitive recipient cells. These experiments proved to be a major challenge, since many parameters needed to be optimized throughout the work. Nevertheless, co-culture systems have proved to be a useful tool to study cell to cell interactions, to test delivery systems and new drug candidates, among others [160-164].

The resazurin assay was performed to assess cellular viability during these experiments. This is a simple, relatively fast and economical method [142]. Moreover, it is believed that resazurin is metabolized in the mitochondria [165], thus ensuring that a possible metabolic activity in the EVs will not interfere with the reading of the fluorescence values.

Therefore, the sensitive cell line (KBM5) was co-cultured with EVs released from its drug resistant counterpart cell line (KBM5-STI), and further treated with STI571. However, to start those experiments, some conditions needed to be tested. Thus, a preliminary study was performed using different incubation periods of KBM5 cells with EVs released from KBM5-STI cells, under different drug treatment durations and different drug concentrations (as explained in **Scheme 1**). The amount of EVs incubated was maintained constant in all plates, being of 5 µg per well. The concentrations of STI571 used were: approximately the IC25, IC50 and IC75 for KBM5 cells in the plates where the cells were in contact with the drug for a longer time (48 h, plates 1 and 3); the IC50, IC75 and IC85 for KBM5 cells in the plates in which cells were treated with drug for 24 h (plates 2 and 4). Those concentrations of STI571 were previously determined, by interpolation of the graph shown in **Figure 11** (**section 1**).



Scheme 1. Description of the workflow of the preliminary co-culture experiment (4 plates) of KBM5 cells with EVs derived from the drug-resistant cell line, KBM5-STI. In all plates, EVs were added only 24 h after seeding the cells, to allow time for the cells to "adapt" to the new environment. Plates' reading was done after 4 h of incubation with resazurin.

From the results shown in **Figure 19**, it was possible to infer that there were no differences among the treatments tested, in what concerns response to STI571. However, this was only a preliminary experiment and important controls were missing from this experiment, such as sensitive cells alone and co-cultured with their own EVs and others which were included in subsequent experiments (indicated below).

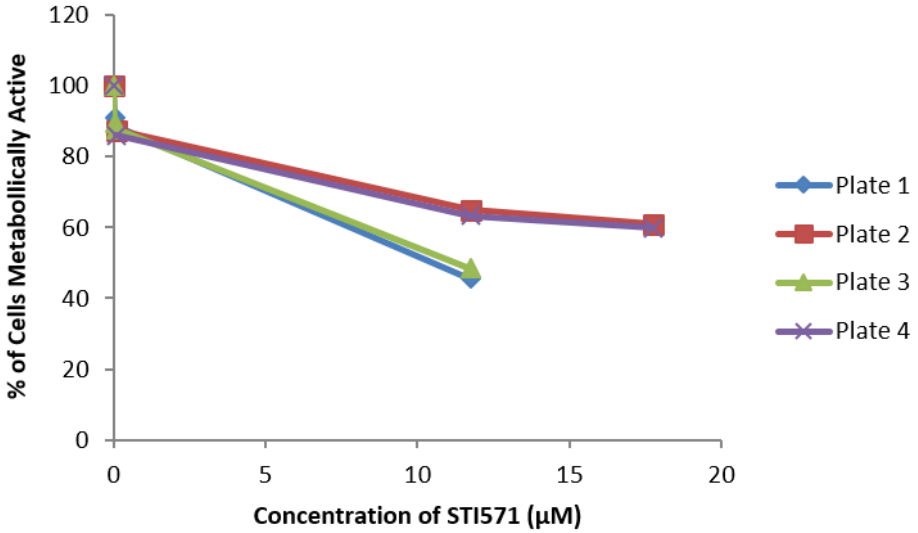


Figure 19. Dose-response curves to STI571, of drug sensitive cells (KBM5) following co-culture with 5 µg of EVs released by KBM5-STI cells. The concentrations of STI571 used were: 0.040 µM, 0.080 µM, 12 µM and 18 µM (approximately the IC25, IC50, IC75 and IC85 for KBM5 cells, respectively). Cells were plated for 24 h and then divided in 4 conditions: Plate 1: 12 h incubation with EVs followed by 48 h incubation with STI571; Plate 2: 12 h incubation with EVs followed by 24 h incubation with STI571; Plate 3: 24 h incubation with EVs followed by 48 h incubation with STI571; Plate 4: 24 h incubation with EVs followed by 24 h incubation with STI571 treatment. Results represent 1 independent experiment only. The concentration of cells seeded was 1.5 x 10^5 cells/mL. The % of cells metabolically active was determined using the resazurin assay (fluorescence read at 530 and 590 nm).

Thus, further experiments were conducted, with the longer incubation periods with EVs (24 h, since longer co-culture would probably be necessary to transfer drug resistance) followed by 24 h drug treatment (since it was believed that the effect would be visible shortly after the co-culture). Important controls were included in these co-culture experiments, such as: KBM5 cells without the presence of EVs (in this case cells incubated with PBS); KBM5 cells with EVs derived from their own cells (KBM5 + EVs S) and KBM5-STI cells only. These controls allowed to study the metabolic activity of the drug sensitive cell line, as well as to understand if EVs could interfere with the normal growth of the cells. In addition, a growth control was included with KBM5 cells without the presence of STI571 but with DMSO (solvent/vehicle of STI571).

As can be seen in **Figure 20**, the co-culture of sensitive cells (KBM5) with EVs released by either of the cell lines (resistant or sensitive to STI571) did not alter the response of the recipient cells to STI571. As expected, KBM5-STI cells were more resistant to STI571 than KBM5 cells. Therefore, under the conditions tested, the EVs released by the drug-resistant cells had no effect on the response of KBM5 cells to STI571.

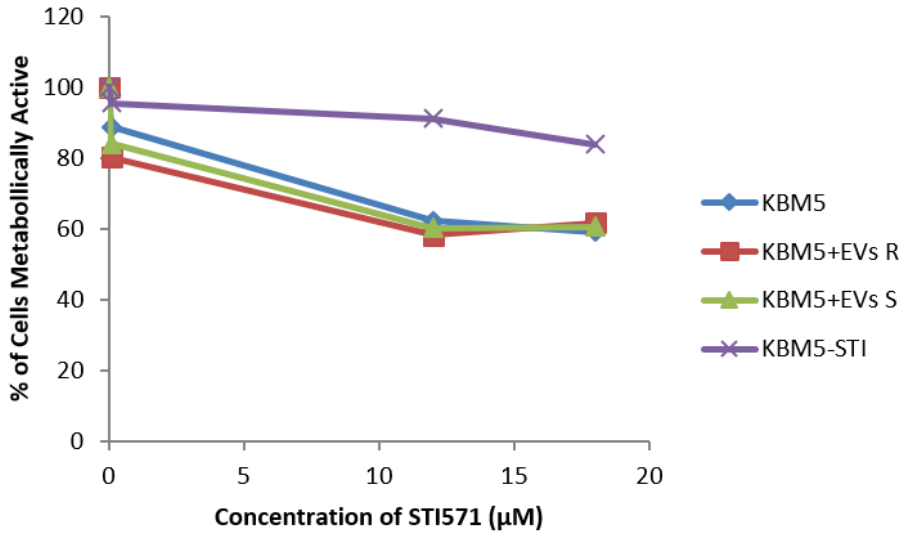
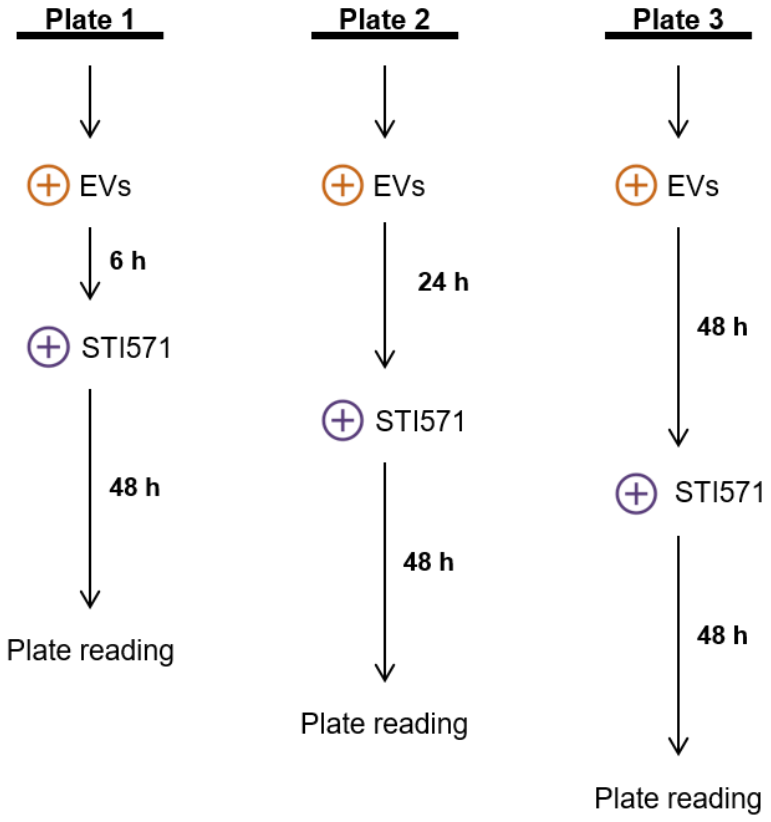


Figure 20. Dose-response curve to STI571 of drug sensitive cells (KBM5) following co-culture with 5 µg of EVs released by KBM5-STI cells (corresponding to the graph line KBM5 + EVs R). The concentrations of STI571 used were: 0.10 µM, 12 µM and 18 µM (approximately the IC50, IC75 and IC85 of KBM5 cells, respectively). Cells were plated for 24 h and then incubated 24 h with EVs followed by 24 h incubation with STI571 treatment. The wells with KBM5 cells only, KBM5 cells incubated with EVs from themselves (EVs S) and KBM5-STI cells only were used as references. Results represent 1 independent experiment only. The concentration of cells seeded was 1.5 x 10^5 cells/mL. The % of cells metabolically active was determined using the resazurin assay (fluorescence read at 530 and 590 nm).

Thereby, other parameters that might influence drug-resistance and not taken into account in the previous experiments were tested in the following experiments. Indeed, other incubation periods of the EVs with sensitive cells (KBM5) were tested (prior to the addition of STI571), such as 6 h, 24 h and 48 h. In addition, STI571 treatment was kept for 48h independently of the co-culture incubation period. The reason for treating cells for 48 h with STI571 is because the IC50 of the STI571 was previously determined at 48 h. Once again, 5 µg of EVs were added per well. The new workflow is shown in **Scheme 2**.



Scheme 2. Description of the workflow of the co-culture experiment (3 plates) of KBM5 cells with EVs derived from the drug-resistant cell line, KBM5-STI. In all plates, EVs were added only 24 h after seeding the cells, to allow time for the cells to "adapt" to the new environment. Plates' reading was done after 4 h of incubation with resazurin.

As shown in **Figure 21**, under all the different condition tested, no effect of the co-culture of EVs released by drug-resistant cells was verified in the sensitive (KBM5) cells. The dose-response curves to STI571 of KBM5 cells and KBM5 cells incubated with EVs (released by either KBM5 or KBM5-STI cells) remained the same. Therefore, under the conditions described above for this experiment, it was again verified that EVs released by the resistant cell line had no effect on the metabolic activity of the sensitive (KBM5) cells under STI571 treatment.

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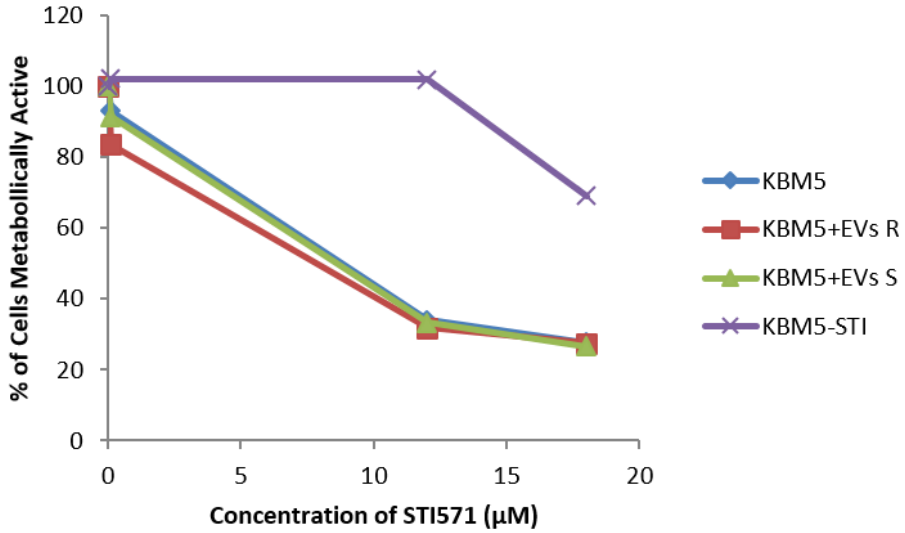

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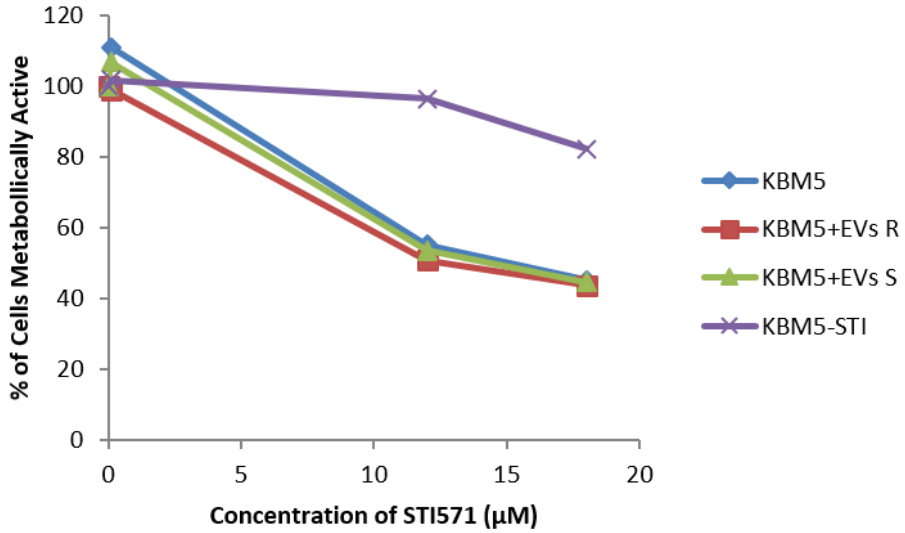

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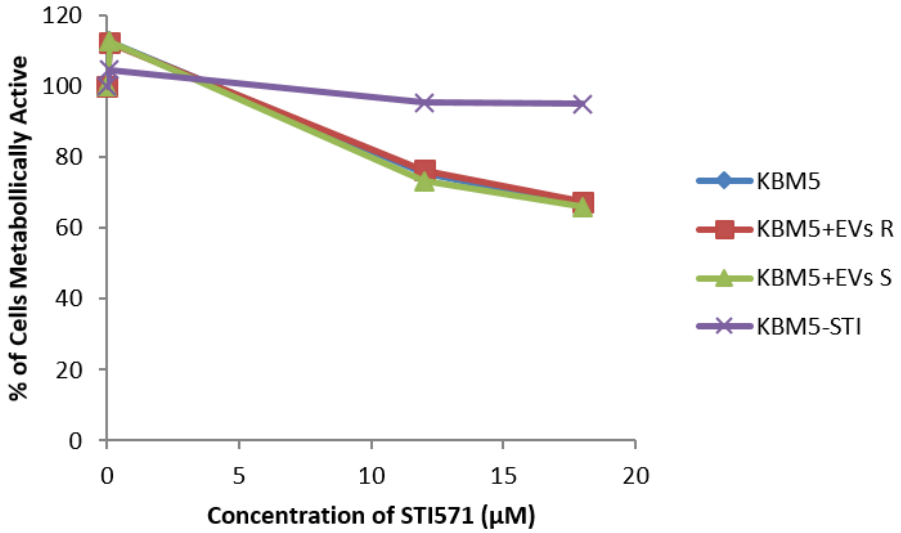

Figure 21. Dose-response curves to STI571 of drug sensitive cells (KBM5) following co-culture with 5 µg of EVs released by KBM5-STI cells for 6 h (A), 24 h (B) and 48 h (C) (corresponding to graph line KBM5 + EVs R). The concentrations of STI571 used were: 0.10 µM, 12 µM and 18 µM (approximately the IC50, IC75 and IC85 of KBM5 cells, respectively). Cells were plated for 24 h and then incubated 6 h, 24 h or 48 h with EVs followed by 48 h incubation with STI571 treatment. The wells with KBM5 cells only, KBM5 cells incubated with EVs released by themselves (EVs S) and KBM5-STI cells only were used as references. Results represent 2 independent experiments. The concentration of cells seeded was 1.5 x 10^5 cells/mL. The % of cells metabolically active was determined using the resazurin assay (fluorescence read at 530 and 590 nm).

One possible explanation for the lack of effect observed during the different conditions tested could be that the cells reached confluency or lack of nutrients during the course of the experiment. For that reason, in order to guarantee that cells were exponentially growing during the course of the experiments, the previous experiments were repeated including a T0 plate, that is, a plate in which cellular metabolic activity was measured at the beginning of the experiment.

Results showed that the magnitude values of fluorescence obtained from the resazurin assay were similar for the plate analyzed at T=0h (T0) and the other plates which were analyzed following different times of incubation (plates 1, 2 and 3). These results suggest that the *plateau* of the fluorescence curve might have been attained during some of the previous experiments (when longer co-culture and drug-treatment times where studied). This means that the fluorescent values read might not have been in the linear response area of the resazurin assay. In summary, these results suggest that the fluorescence values obtained in the previous experiments might not have been directly correlated with the metabolic activity of cells. Indeed, during the course of this work, a new batch of the resazurin reagent was purchased, which might have provided different

fluorescent readings from the initial batch. Therefore, the initially optimized conditions (optimized by other members of the team prior to the initiation of this work) for the resazurin assay might not have been maintained throughout some of the previous experiments.

Thus, in order to decrease the fluorescence readings of the cells in the resazurin assay and, in this manner, to guarantee that the work was performed during the exponential growth phase of the cells, optimization of the conditions for the resazurin assay was again performed. The ideal concentration of cells to be seeded (not reaching the plateau of fluorescence values by the end of the co-culture experiment) was studied, using the new batch of resazurin reagent and up to 120 h of total experiment duration (i.e. total duration of cells in culture). Since the previous experiments were started with 1.5×10^5 cells/mL (which was the previously optimized conditions for the previous resazurin batch and experiments for up to 48 h of duration), lower concentrations of cells were tested.

Results are presented in **Figure 22**, which indicate that, for both cell lines, the concentration of cells used in the previous experiments (1.5×10^5 cells/mL), in which the total duration of the experiments was as long as 96 h (Plate 2) or 120h (Plate 3), were at the beginning of the *plateau* phase i.e. at the limit of the linearity of the assay. Thus, there was a possibility that the previously observed lack of effect of the EVs released by drug-resistant cells in the drug sensitive cells was due to this methodological problem, but only when the duration of the experiments was as long as 96h or 120h. Therefore, results from **Figure 22** indicate that the ideal cell density to be used in co-culture experiments of up to 96 or 120h should be 8.0×10^4 cells/mL. This cell density allows cells to be at the exponential phase of the growth curve during the total duration of the experiment.

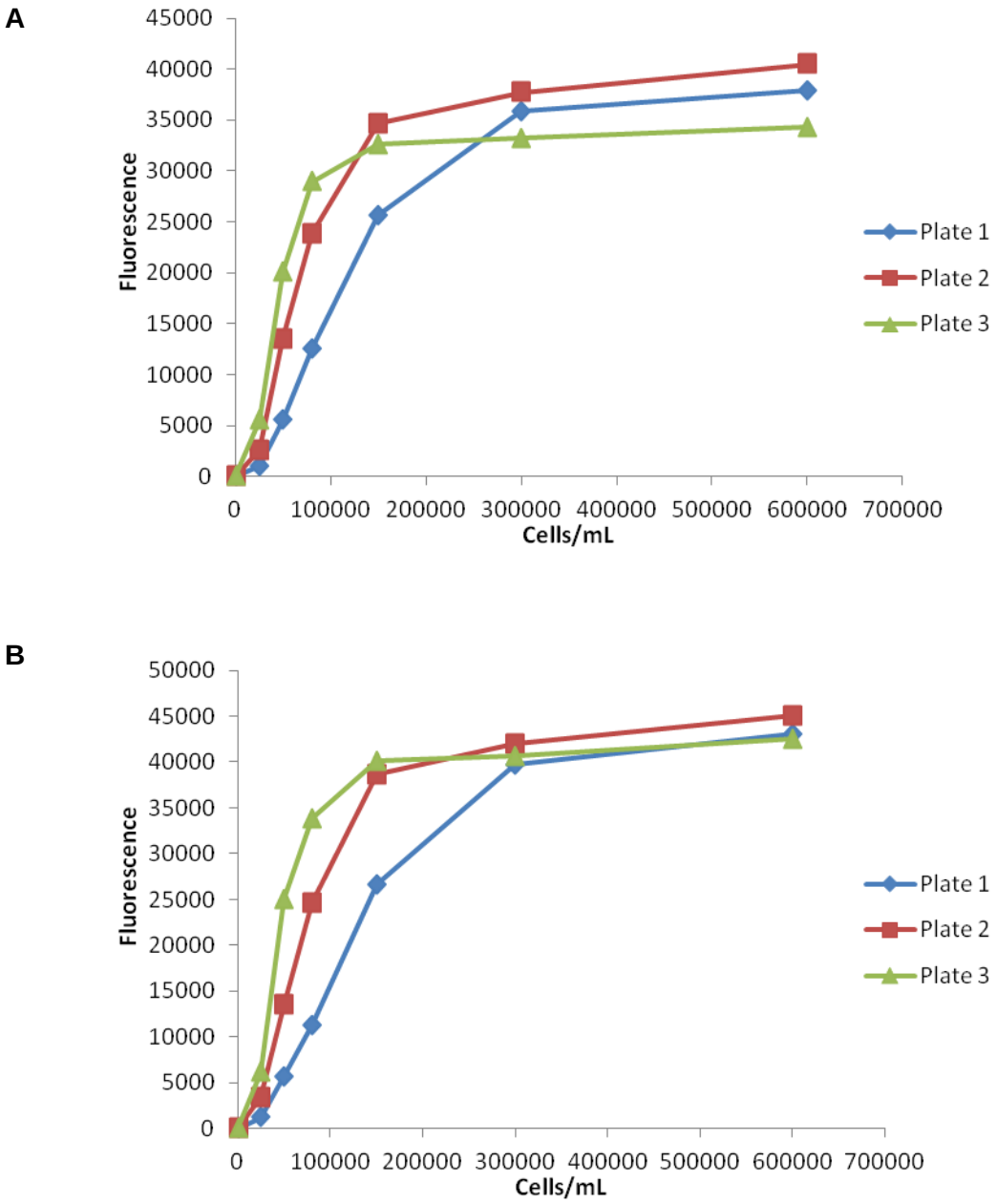

Figure 22. Optimization of the ideal concentration of cells to be seeded of KBM5 (A) and KBM5-STI (B) cells in a 96-well plate during the total duration of co-culture experiments. Total time in culture – Plate 1: 78 h, Plate 2: 96 h, Plate 3: 120 h. Results were obtained using the resazurin assay (fluorescence read at 530 and 590 nm). The concentrations tested were the following: 2.5 x 10^4, 5.0 x 10^4, 8.0 x10^4, 1.5 x 10^5, 3.0 x 10^5 and 6.0 x 10^5 cells/mL. Results represent 1 independent experiment only. Results were further confirmed by trypan blue exclusion assay and flow cytometry (data not shown).

Therefore, further co-culture experiments were conducted, by seeding 8.0 x 10^4 cells/mL, and decreasing the concentration of STI571 to 6.2 µM, 3.1 µM and 0.20 µM, (this last concentration being close to the IC50 of KBM5 cells). In addition, new aliquots of STI571 were prepared (to avoid possible drug instability problems). The workflow of the co-culture

experiment was maintained as before, represented in **Scheme 2**, but including the alterations referred above (number of cells plated and new aliquots of STI571).

In spite of the methodological alterations performed, the obtained results (**Figure 23**) showed that EVs released by KBM5-STI cells had no effect on the percentage of KBM5 cells metabolically active in the presence of STI571, i.e. had not effect of the response of the sensitive cells to STI571. Thus, these results were in agreement with the previously obtained results (**Figures 20 and 21**). Moreover, and surprisingly, it was possible to observe that the drug response of the resistant (KBM5-STI) cells was closer to the drug response of the sensitive (KBM5) cells in the longer co-culture experiment (Plate 3). Even though this result would need to be repeated in order to be confirmed, it is possible that this was caused by the longer incubation period of this plate. For this reason, in further experiments Plate 3 was removed from the study.

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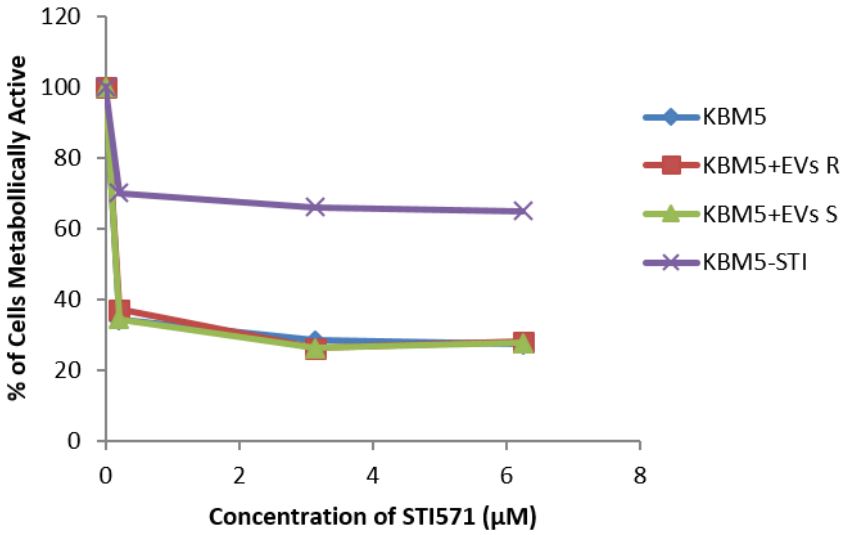

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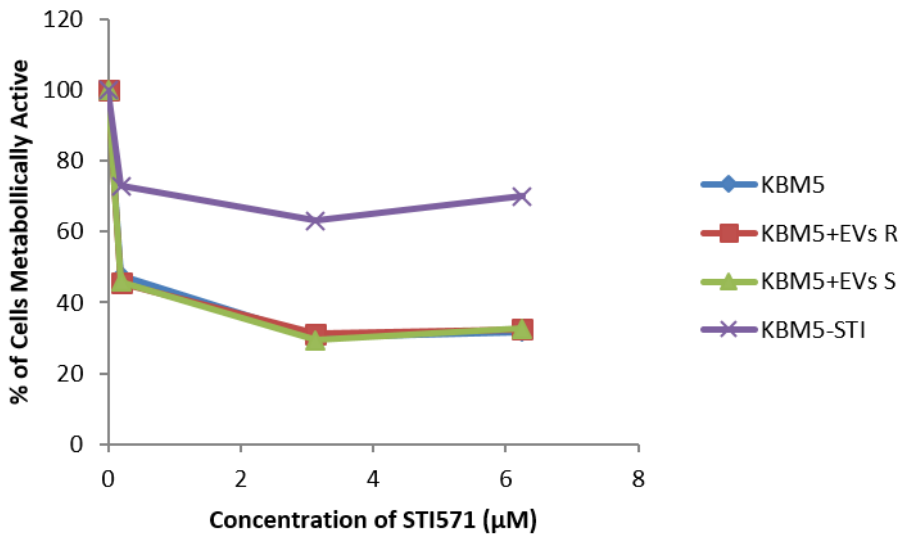

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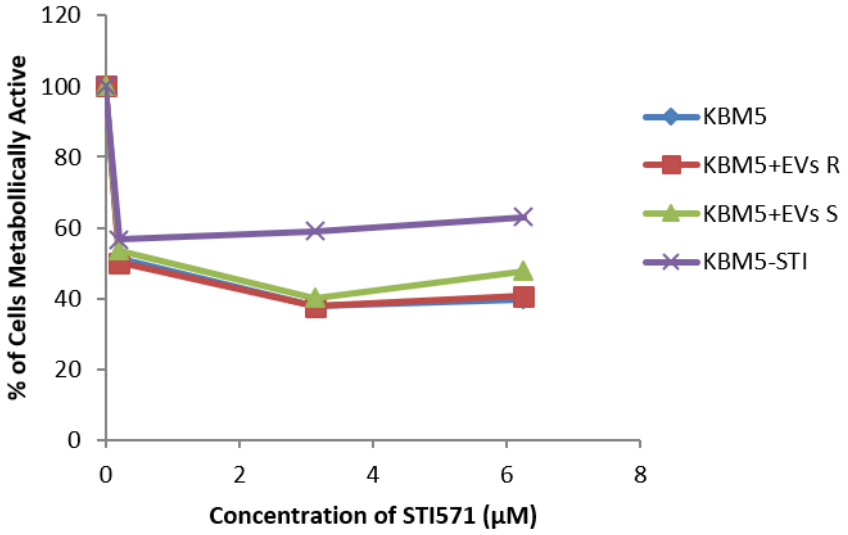
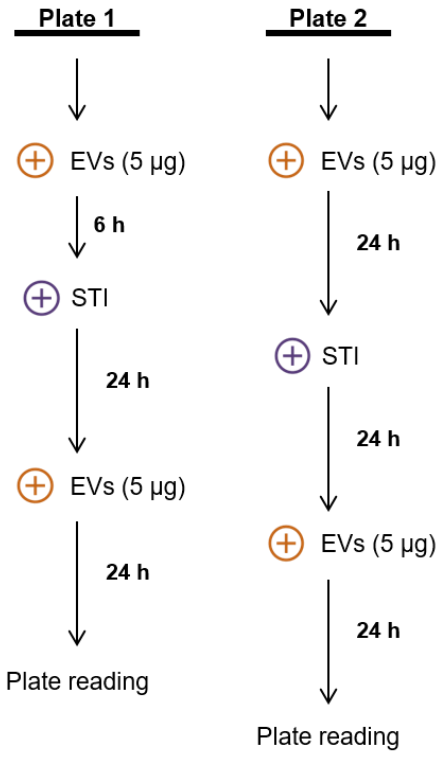


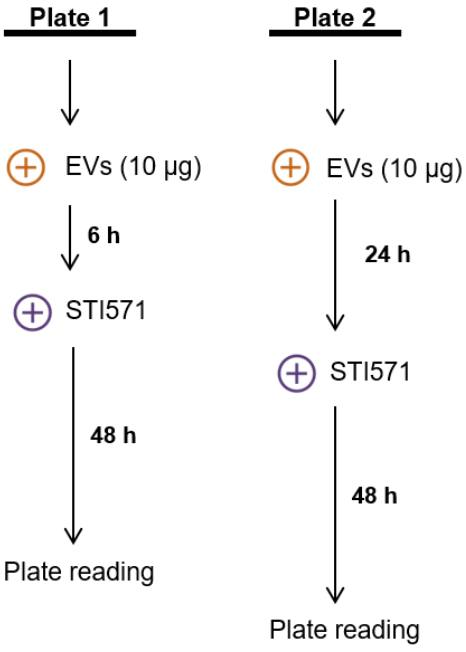
Figure 23. Dose-response curves to STI571 of drug sensitive cells (KBM5) following co-culture with 5 µg of EVs released by KBM5-STI cells for 6 h (A), 24 h (B) and 48 h (C) (corresponding to graph line KBM5 + EVs R). The concentrations of STI571 used were: 0.20 µM, 3.1 µM and 6.2 µM. Cells were plated for 24 h and then incubated 6 h, 24 h or 48 h with EVs followed by 48 h incubation with STI571 treatment. The wells with KBM5 cells only, KBM5 cells incubated with EVs released by themselves (EVs S) and KBM5-STI cells only were used as references. Results represent 1 independent experiment only. The concentration of cells seeded was 8.0 x 10^4 cells/mL. The % of cells metabolically active was determined using the resazurin assay (fluorescence read at 530 and 590 nm).

In an attempt to test more possible co-culture conditions and to try to better mimic the continuous effect (of EVs exposure) which might happen in the human body, 5 µg of EVs per well were added not only before STI571 treatment but also after drug treatment (**Scheme 3**). In this manner, a more frequent co-culture with EVs might better resemble the real conditions in the human body. Moreover, since the BCR-ABL protein has a half-life above 24h [166], these new conditions would ensure that the BCR-ABL from the EVs (probably both wild-type and mutated, the last conferring resistance to the cells) was more frequently co-incubated with the sensitive cells and, thus, would be present during the duration of the drug treatment. In addition, these new conditions allowed more EVs to be in co-culture with the recipient cells.



Scheme 3. Description of the general workflow of the co-culture experiment (2 plates) of KBM5 cells incubated with EVs released by the drug-resistant cell line, KBM5-STI. In both plates, the first 5 µg of EVs were added 24 h after seeding the cells, to allow time for the cells to "adapt" to the new environment. Plates' reading was done after 4 h of incubation with resazurin.

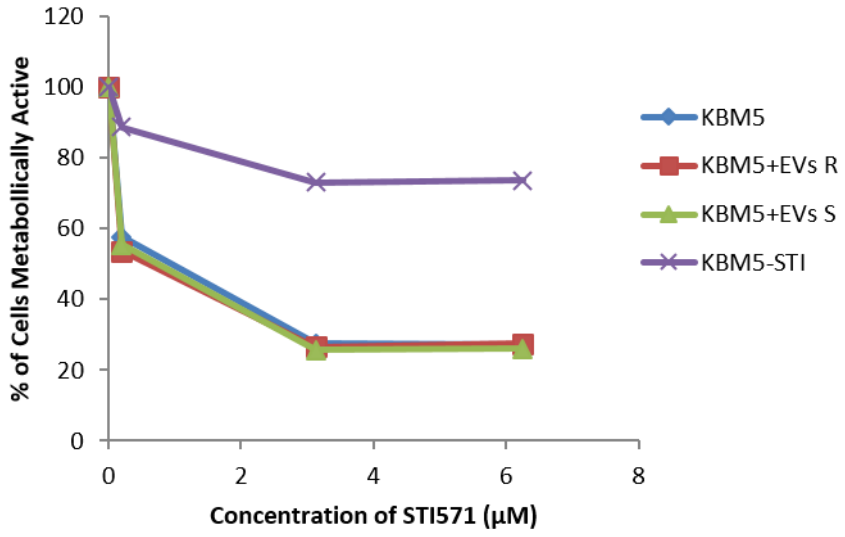
Additionally, the effect of co-culturing 10 µg of EVs (from drug resistant cells) with the sensitive cells 6 h and 24 h prior the drug treatment was also tested (**Scheme 4**).



Scheme 4. Description of the general workflow of the co-culture experiment (2 plates) of KBM5 cells incubated with EVs released by the drug-resistant cell line, KBM5-STI. In both plates, 10 µg of EVs were added 24 h after seeding the cells, to allow time for the cells to "adapt" to the new environment. Plates' reading was done after 4 h of incubation with resazurin.

The obtained results (**Figures 24 and 25**) showed that EVs released by the drug resistant cells had no effect in the percentage of KBM5 cells metabolically active in the presence of STI571, i.e. had no effect in the drug response of the sensitive cells. This lack of effect was observed when EVs from drug resistant cells were more frequently co-cultured with the sensitive cells, both before and after drug treatment (**Figure 24**), and when the quantity of EVs co-cultured was increased to the double (10 µg of EVs before drug treatment; **Figure 25**).

A



B

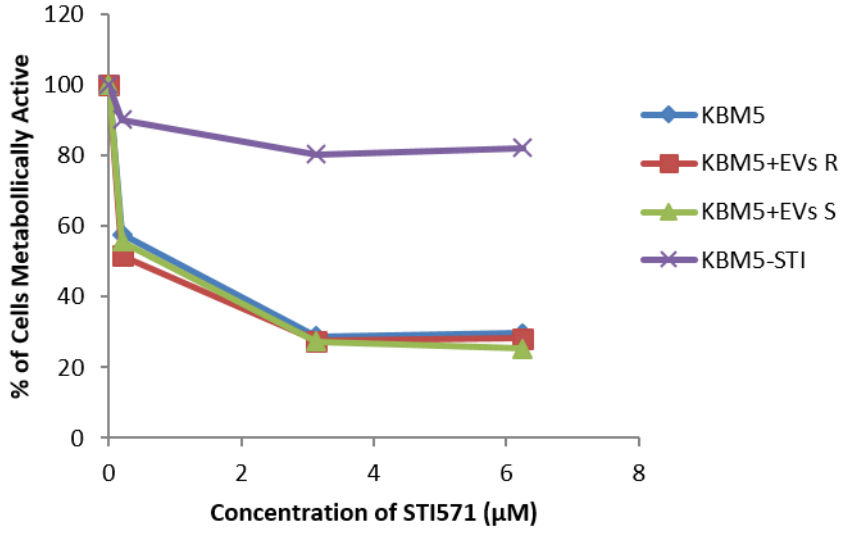
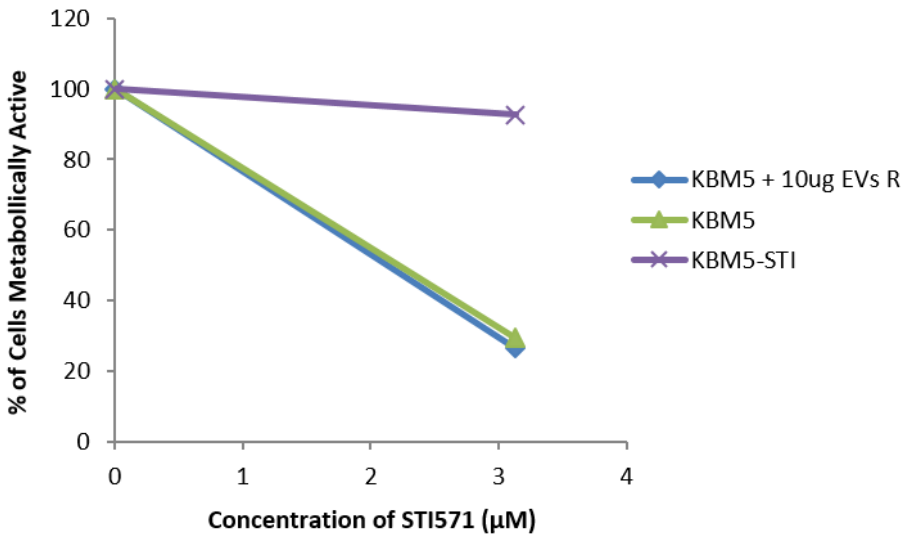


Figure 24. Dose-response curves to STI571 of drug sensitive cells (KBM5) following co-culture with 10 µg (5 µg + 5 µg) of EVs released by KBM5-STI cells (corresponding to graph line KBM5 + EVs R). 5 µg of EVs were added for 6 h (A) and 24 h (B) before STI571 treatment, and 5 µg were added again 24 h after the addition of STI571. The concentrations of STI571 used were: 0.20 µM, 3.1 µM and 6.2 µM. The wells with KBM5 cells only, KBM5 cells incubated with EVs released by themselves (EVs S) and KBM5-STI cells only were used as references. Results represent 1 independent experiment only. The concentration of cells seeded was 8.0 x 10^4 cells/mL. The % of cells metabolically active was determined using the resazurin assay (fluorescence read at 530 and 590 nm).

A



B

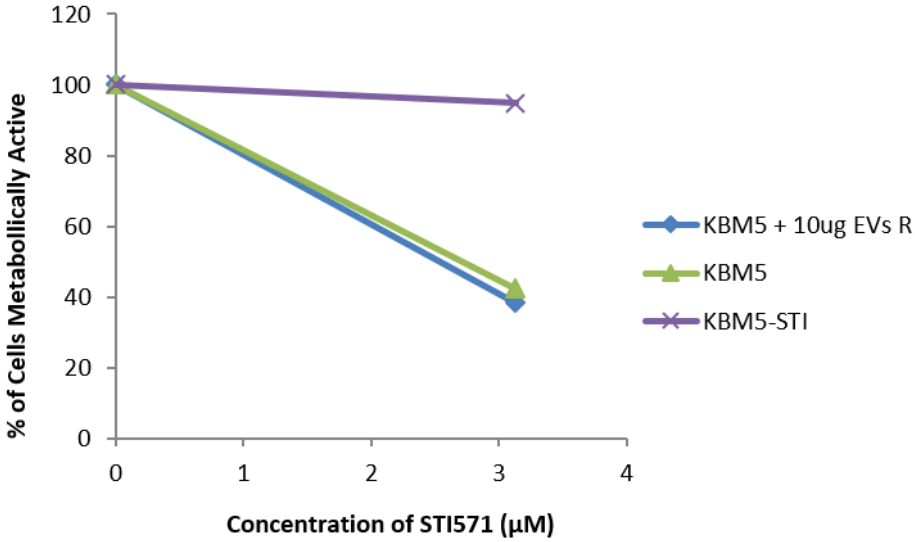
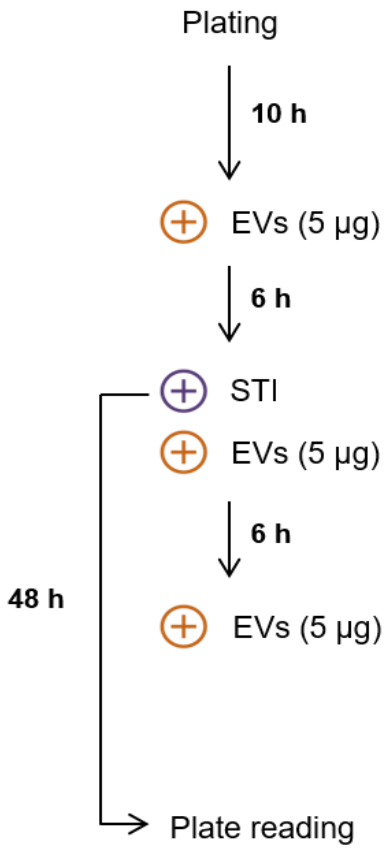


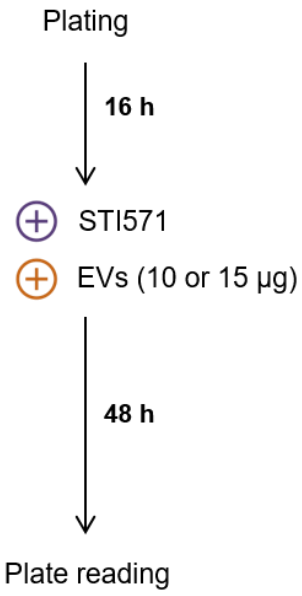
Figure 25. Dose-response curves to STI571 of drug sensitive cells (KBM5) following co-culture with 10 µg of EVs released by KBM5-STI cells. 10 µg of EVs were added 6 h (A) and 24 h (B) before STI571 treatment (corresponding to graph line KBM5 + 10 µg EVs R). The concentration of STI571 used was 3.1 µM. The wells with KBM5 cells and KBM5-STI cells only were used as references. Results represent 1 independent experiment only. The concentration of cells seeded was

8.0 x 10^4 cells/mL. The % of cells metabolically active was determined using the resazurin assay (fluorescence read at 530 and 590 nm).

The last approach attempted in this work involved the addition of EVs released by KBM5-STI cells before, together with and after STI571 treatment (3 x 5 µg per well, i.e. a total of 15 µg of EVs added to the sensitive cells; **Scheme 5**). In addition, the intervals between the additions of EVs to the sensitive cells were slightly shortened, as well as the time allowed for cells to "adapt" to the new wells (after seeding and before co-culture with EVs). This new approach also allowed to test the co-culture with EVs (10 µg and 15 µg) together with the STI571 treatment (**Scheme 6**).



Scheme 5. Description of the general workflow of the co-culture experiment of KBM5 cells with EVs released by the drug-resistant cell line, KBM5-STI. The EVs (5 µg) were added 10 h after seeding the cells, so that the cells could "adapt" to the new environment, as well as during and 6 h after STI571 treatment. Plate reading was done after 4 h of incubation with resazurin.



Scheme 6. Description of the general workflow of the co-culture experiment of KBM5 cells with EVs released by the drug-resistant cell line, KBM5-STI. The EVs (10 and 15 µg) were added 16 h after seeding the cells, so that the cells could "adapt" to the new environment. Plate reading was done after 4 h of incubation with resazurin.

The results obtained are presented in **Figures 26 and 27** and demonstrated that there were no alterations on the metabolic activity of sensitive (KBM5) cells following STI571 treatment, i.e. of response to STI571, following incubation with a total of 15 µg of EVs released by the drug-resistant cells, when compared with KBM5 cells without co-culture of EVs or with KBM5 cells incubated with EVs released by themselves. The same negative results were obtained when either 10 µg or 15 µg of EVs were added together with the STI571 treatment.

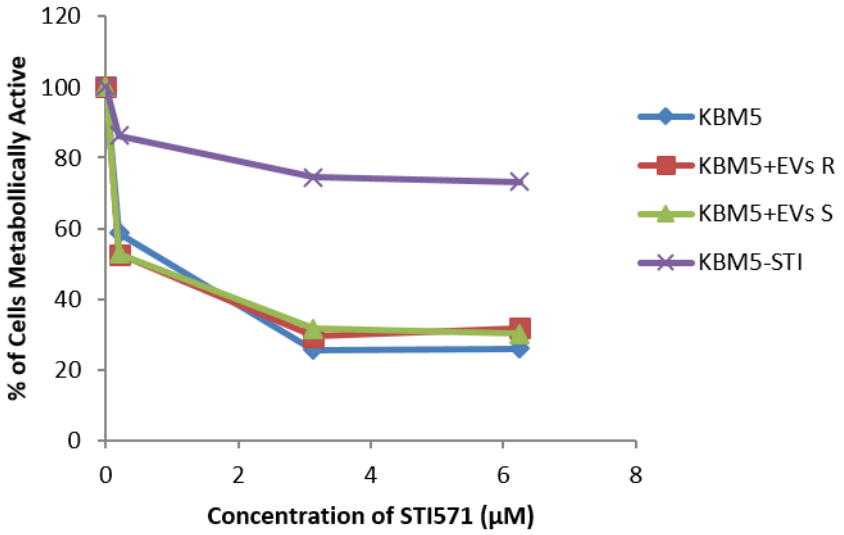


Figure 26. Dose-response curve to STI571 of drug sensitive cells (KBM5) following co-culture with 15 µg (5 µg + 5 µg + 5 µg) of EVs released by KBM5-STI cells (corresponding to graph line KBM5 + EVs R). 5 µg of EVs were added: 6 h before, together with and 6 h after drug treatment. The concentrations of STI571 used were: 0.20 µM, 3.1 µM and 6.2 µM. The wells with KBM5 cells only, KBM5 cells incubated with EVs released by themselves (EVs S) and KBM5-STI cells only were used as references. Results represent 1 independent experiment only. The concentration of cells seeded was 8.0 x 10^4 cells/mL. The % of cells metabolically active was determined using the resazurin assay (fluorescence read at 530 and 590 nm).

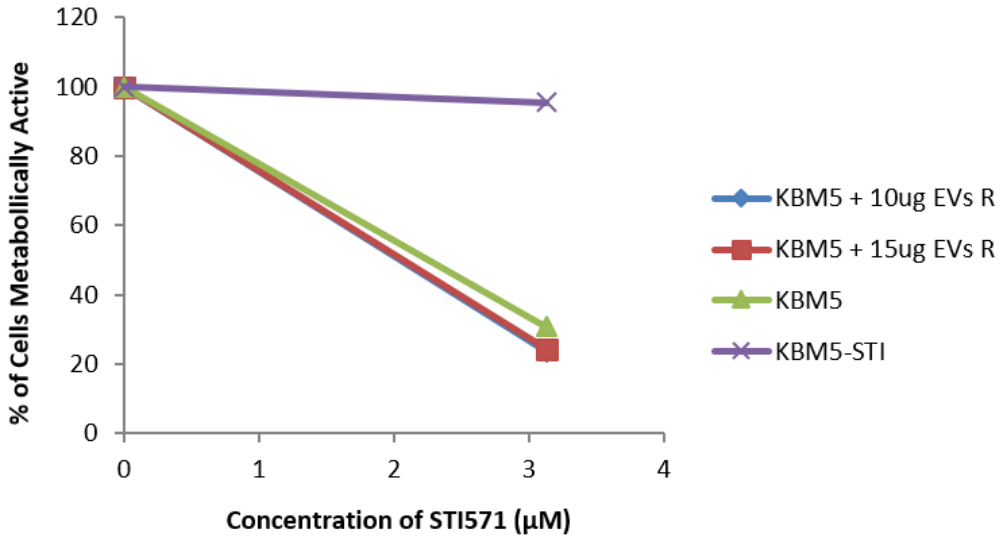


Figure 27. Dose-response curve to STI571 of drug sensitive cells (KBM5) following co-culture with 10 and 15 µg of EVs released by KBM5-STI cells. 10 or 15 µg of EVs were added together with STI571 (corresponding to graph lines KBM5 + 10 µg EVs R and KBM5 + 15 µg EVs R, respectively). The concentration of STI571 used was 3.1 µM. The wells with KBM5 cells and KBM5-STI cells only were used as references. Results represent 1 independent experiment only. The concentration of cells seeded was 8.0 x 10^4 cells/mL. The % of cells metabolically active was determined using the resazurin assay (fluorescence read at 530 and 590 nm).

The results from all the co-culture experiments described in this thesis are summarized in **Table 4**. From these results we may infer that, in the cell lines studied and under the conditions studied, although BCR-ABL is present in the EVs released by donor drug resistant cells (as confirmed by the BCR-ABL protein and mRNA content analysis), this may not have impact on the resistance of recipient drug sensitive cells to STI571.

Table 4. Summary of the co-culture experiments stating similarities and differences among them.

Figure	[Cells] (cells/mL)	[EVs] (µg)	[STI571] (µM)	Time of incubation with EVs (before STI571 treatment) (h)	Duration of STI571 treatment (h)
20	1.5 x 10^5	5	0.10; 12; 18	24	24
21	1.5 x 10^5	5	0.10; 12; 18	6, 24 and 48	48
23	8.0 x 10^4	5	0.20; 3.1; 6.2	6, 24 and 48	48
24 and 25	8.0 x 10^4	10	0.20; 3.1; 6.2	6 and 24	48
26 and 27	8.0 x 10^4	15	0.20; 3.1; 6.2	6	48

There are possible explanations for what might be happening at the molecular level. Firstly, it is possible that the recipient cells do not take up the EVs from the donor cells. Secondly, since it was not possible to verify if the BCR-ABL in the EVs was wild-type or mutated, it is possible that only wild-type BCR-ABL is present in the EVs. Thirdly, it is possible that if BCR-ABL is transferred to recipient cells, it may be non-functional in the recipient cells (thus not contributing to a drug-resistant phenotype in recipient cells). Finally, the EVs released by drug-resistant cells might not shed enough mutant BCR-ABL (mRNA and/or protein) to alter the drug response of recipient sensitive cells. All these possibilities could lead to the verified undetectable influence of the EVs released by drug-resistant cells on the drug response of sensitive recipient cells.

However, other experimental conditions should be tested before taking final conclusions. For example, high fluorescence values were always read on the resazurin assay, even after decreasing the cell density of seeded cells. One possible reason for this is the time of incubation with resazurin (4 h), which may not have been the ideal (this time usually depends on cell growth). Therefore, the incubation time with resazurin could have been optimized for the cell lines used in this work and for the necessary duration of the experiments. In fact, the incubation time with resazurin varies between 1 h to 4 h, depending on the cell concentration and cell type (because of possible differences in the cellular rate

of proliferation). When a high number of cells are incubated with resazurin, for long periods, the fluorescent product of resazurin reduction, the resorufin, is in turn reduced to a non-fluorescent product called hydro-resorufin [142]. Thereby, the value of fluorescence read will not reflect the number of cells present in the well. Therefore, the protocol for the resazurin assay should in future experiments be adapted to the cell lines and duration of the experiments necessary for this specific work.

Another possible alteration to the co-culture experimental conditions is to further increase the amount of EVs added to each well. Although 3 different concentrations of EVs were tested, the proportion of EVs/cells may still need to be optimised. Nonetheless, it should be a compromise to balance this increase with the amount of EVs that would be biologically acceptable for the recipient cells. However, to our knowledge, there is no information on the literature on what would be considered physiological (or even pathological) concentrations of EVs delivered to recipient cells. In addition, an increase in the amount of EVs to be co-cultured would be technically quite difficult, requiring an even bigger number of donor (resistant) cells in culture for isolation of EVs.

In summary, the co-culture experiments should be further optimized in order to allow definite conclusions. However, our preliminary results indicate that, under the conditions tested, there is no transfer of drug resistance mediated by the EVs released by these drug resistant cells to drug sensitive cells.

Given the results here obtained, regarding the presence of BCR-ABL in the EVs shed by these leukemic cells, it would be interesting to confirm if those EVs (from either drug sensitive or drug resistant cells) may transfer BCR-ABL to non-tumor cells. Indeed, other authors have shown that BCR-ABL might be transferred to neutrophils and HEK293 cells, human embryonic kidney cells, by contact with EVs from CML cells. Additionally, the same authors showed that this shedding of EVs containing BCR-ABL has physiological significance, since BCR-ABL present in EVs influenced the function of recipient cells. Furthermore, the injection of those EVs in immunodeficient mice had a strong impact, causing CML-like symptoms in those mice. Thus, this phenomenon might have an important role in tumor initiation and progression [158, 159]. Besides, another group has demonstrated that endothelial cells acquire both BCR-ABL oncoprotein and mRNA, after incubation with EVs released by BCR-ABL$^+$ cells (either from plasma of recently diagnosed CML patients or from a CML cell line, K562) [167].

Finally, the results here presented show that the EVs released by both (drug sensitive and drug resistant) cell lines have BCR-ABL on their cargo. In addition, the data presented also suggest that there is selective packaging of this oncogene into the EVs shed

by these cells. Unfortunately, it was not possible to sequence the mRNA or the protein in the EVs to confirm if they were wild-type or mutated. Future work will continue this study since there are obvious implications for the possible detection of BCR-ABL in circulating EVs from liquid biopsies of chronic myeloid leukemia patients. Indeed, the presence of this biomarker in circulating EVs may allow a more sensitive detection of BCR-ABL since each tumor cell sheds many more EVs. In addition, the cargo of the EVs is more stable from degradation by nucleases or proteinases [117], making EVs an optimal source of cancer biomarkers.

IV. Conclusion

4.1. Concluding Remarks

The success of TKIs in treating CML highlighted the potential of targeting oncogenic kinases with small molecules. The discovery of STI571 caused a dramatically improvement in CML patients' prognosis, transforming an irrevocably lethal disease into a paradigm of molecular target therapy [47]. Nonetheless, there is a relative high relapse rate following treatment with this drug, caused by resistance of leukemic cells to the drug. This drug resistance may have various causes or mechanisms, representing a serious clinical drawback [36, 56]. Hence, it is urgent to clearly identify mechanisms behind resistance of leukemic cells to STI571, in order to prevent this problem and to find new therapeutic targets that may contribute to counteract this very serious clinical problem.

Many studies have suggested that EVs may contribute to the dissemination of cancer drug resistance, by transferring drug resistance traits from donor drug-resistant cells to sensitive cells [106, 138]. Indeed, it is known that the cargo of EVs released by drug resistant cancer cells contains proteins, microRNAs, mRNAs and other molecules from the donor cells and that this cargo is functional in recipient drug sensitive cells, being capable of altering the phenotype of recipient cells towards drug resistance [168, 169]. This is an emerging area of interest with possible impact on identification of novel approaches to predict and counteract drug resistance [117].

This work aimed at verifying if: i) EVs released by BCR-ABL positive leukemia cell lines had BCR-ABL (protein and mRNA) on their cargo; and ii) if drug resistance could be transferred from leukemic drug resistant cells (with mutant *BCR-ABL*) to leukemic drug sensitive cells (with wild-type BCR-ABL).

In this work, the drug response profiles of a pair of drug sensitive (with wild-type BCR-ABL) and drug resistant (with mutant BCR-ABL) counterpart cell lines were initially confirmed (using the resazurin assay). By determining the dose-response curves and comparing the IC50 of STI571 in both cell lines, it was possible to confirm that KBM5-STI cells were resistant to STI571 (up to high concentrations of this drug) whereas KBM5 cells were sensitive to this drug.

The characterization of the EVs isolated from this pair of cell lines allowed to confirm their successful isolation using an ultracentrifugation protocol. Indeed, DLS, NTA and TEM analysis confirmed that the obtained size-ranges were the ones expected for EVs (10 – 1000 nm). The cup shaped morphology, typical of EVs, was also verified by TEM. Furthermore, by WB analysis, the presence of several EVs markers (such as CD63, HSP70

and Flotillin-1) and the absence of cellular contaminants were confirmed. Additionally, it was possible to verify that EVs released by both cell lines (sensitive and resistant to STI571) presented similar sizes and protein markers.

Moreover, the BCR-ABL content was studied both on the counterpart cell lines and on the EVs released by those cell lines. Surprisingly, the results here obtained (by WB) suggest that the BCR-ABL protein is selectively packaged into EVs, providing a mechanism for oncoprotein shedding. In addition, preliminary results obtained as collaboration with Centro Hospitalar São João also suggest that the mRNA of BCR-ABL is packaged into those EVs. However, the presence of the mRNA needs to be further confirmed.

The work presented in this thesis also investigated if there was a transfer of drug resistance mediated by EVs, from the drug resistant to the drug sensitive counterpart cells. For that, co-culture experiments were performed, based on the incubation of drug-sensitive cells (KBM5) with EVs released by drug-resistant cells (KBM5-STI). The EVs from donor cells were in contact with the recipient cells during different periods and in different concentrations; different times were also tested for addition of EVs to recipient cells. The dose-response curves to STI571 of the recipient cells (with or without incubation with EVs from donor cells) were assessed, in order to ascertain if KBM5 cells acquired drug resistance traits. Results from the different experiments carried out, under the different treatment times and conditions tested, did not show evidence for a transfer of drug resistance from resistant to sensitive cells. However, it is possible that under different experimental conditions the results obtained would be different. Unfortunately, due to technical and time constraints, it was not possible to verify if the BCR-ABL present in the EVs was wild-type or mutated.

In summary, the main conclusion drawn from this work was that BCR-ABL is packaged into the cargo of EVs released by BCR-ABL^{+} leukemia cells (both sensitive and resistant to STI571). Nevertheless, although this transfer may be mediated by EVs released by drug-resistant cells, with mutant *BCR-ABL*, incubation of drug sensitive cells with those EVs did not influence drug response of recipient cells, under the conditions tested. More studies are required in order to investigate the relevance of BCR-ABL packaging into EVs and its impact in the progression of disease in drug resistance.

4.2. Future perspectives

Firstly, in order to confirm the results obtained from the co-culture experiments, more conditions should be tested regarding the duration of cellular incubation with resazurin, duration of cellular incubation with EVs and concentration of EVs added, as previously mentioned.

Secondly, it is essential to sequence the mRNA of BCR-ABL present in the EVs released by drug resistant cells, in order to verify if it is wild-type and/or mutated.

Furthermore, it would be interesting to investigate the presence of DNA of BCR-ABL in those EVs. Indeed, increasing published evidence shows the presence of fragments of DNA on the cargo of EVs. In particular, DNA of BCR-ABL was recently found in EVs released by K562 cells [158, 159]. Accordingly, it would be interesting to verify the packaging of DNA coding for BCR-ABL into the EVs from the cell lines used in this study.

Moreover, it would be interesting to test if the EVs from the cell lines studied may transfer BCR-ABL to BCR-ABL$^-$ cells (to other tumor cells but non-leukemic or even to non-tumor cells). This would be a *proof of concept* of the intercellular transfer of BCR-ABL and would allow studying the functional implications on recipient cells.

Finally, it would be very interesting to verify if it is possible to detect BCR-ABL in the EVs isolated from CML patients' plasma, as biomarkers of this disease. In fact, circulating EVs from liquid biopsies of hematological diseases have been studied with the purpose of finding more sensitive and earlier diagnostic and prognostic methods [170]. This would be a translational application of the present study, allowing transfer of knowledge into the clinic, contributing to early diagnosis and to personalized therapeutic decisions and, thus, possibly having impact in CML patients' survival.

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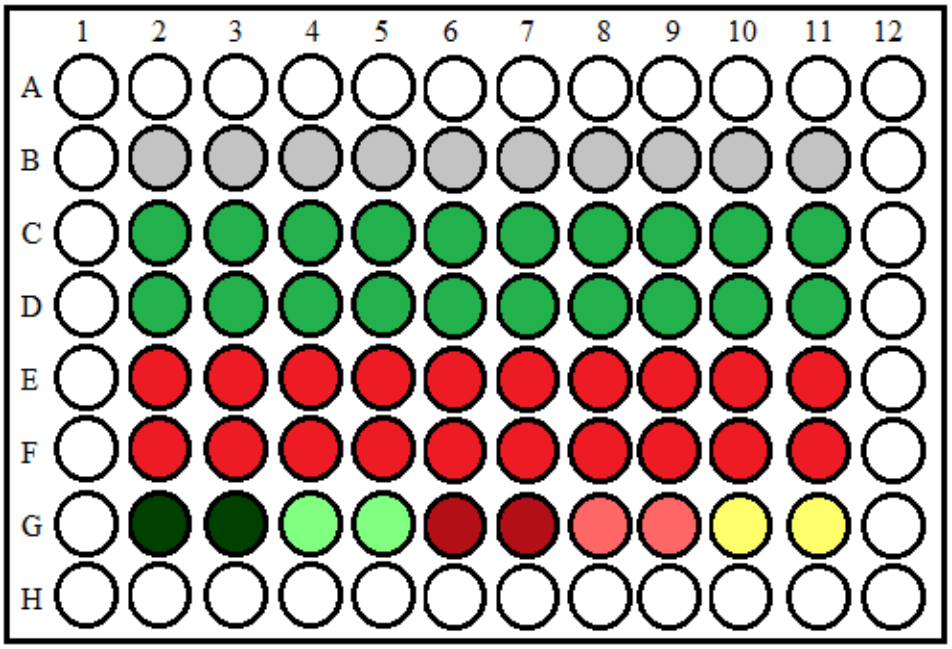
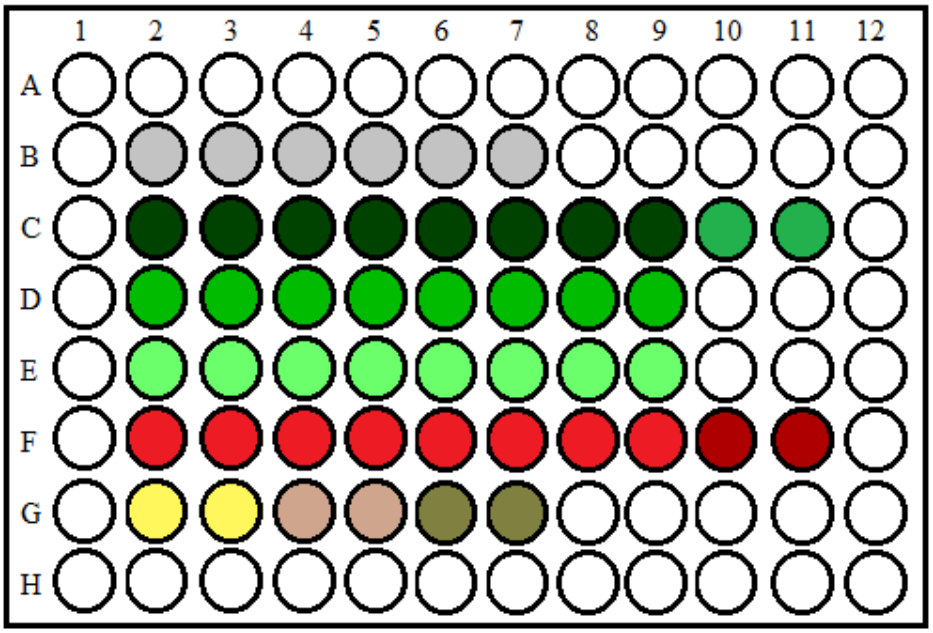
VI. Annexes

Annex 1



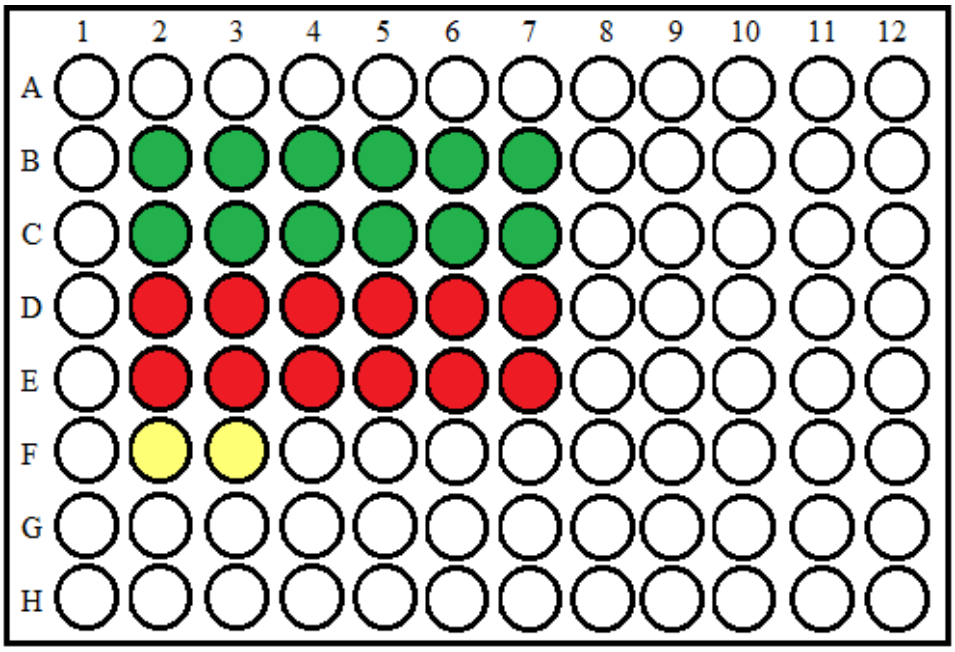
Annex 1. Schematic representation of a 96-well plate for determination of the IC50 of the imatinib mesylate (STI571). Green and red colors represent the KBM5 and KBM5-STI cell lines, respectively. Both cell lines were treated with different STI571 concentrations (columns 2-11, lines C-F): 0.04 µM, 0.08 µM, 0.15 µM, 0.31 µM, 0.63 µM, 1.3 µM, 2.5 µM, 5.0 µM, 10 µM and 20 µM. Dark green and dark red wells (line G: rows 2, 3 and 6, 7) correspond to the KMB5 and KBM5-STI cell lines, respectively, cultured with the higher percentage of DMSO used (equivalent to the one used when treating cells with 20 µM of STI571, which is nontoxic, <0.25% per well). Light green and light red wells represent the KBM5 and KBM5-STI cells in culture medium only, respectively (line G: rows 4, 5 and 8, 9). These 8 wells mentioned (line G from well 2 to well 9) represent a cell proliferation control. Yellow wells contained normal culture medium only (Blank). The gray color represents the different drug concentrations without the presence of the cells (background). Remaining white wells contained PBS or medium to maintain humidity on the plate.

Annex 2



Annex 2. General schematic representation of a 96-well plate for the co-culture of recipient sensitive cells with EVs released by drug-resistant cells. Green and red colors represent, the KBM5 and KBM5-STI cell lines, respectively. Different shades of each green and red colors represent the following: Line C (row 2-9): KBM5 cells with addition of PBS; Line D (row 2-11): KBM5 cells with addition of 5 µg of EVs derived from KBM5-STI cells; Line E (row 2-11): KBM5 cells with addition of 5 µg of EVs derived from KBM5 cells. Gray color represents the different drug concentrations without the presence of the cells (background). In the two first experiments: 0.1 µM of STI571 was added to rows 2 and 3; 12 µM was added to rows 4 and 5; 18 µM (from line B to F) was added to rows 6 and 7. In the following experiments, the concentrations of STI571 used were 0.2 µM, 3.13 µM or 6.25 µM. Cells from rows 8 and 9 were cultured with the higher percentage of DMSO used (corresponding to the one used when treating cells with 18 µM STI571 or 6.25 µM, which is nontoxic, <0.25% per well), as a control. Regarding columns 10 and 11, cells from C and F lines were cultured in normal cultured medium during the experiment. Wells from line G did not contain cells. Yellow wells contained normal culture medium only, the following wells contained just PBS and medium (line G, rows 4 and 5) and the two other wells contained medium, PBS and DMSO at its higher concentration in the experiment (line G, rows 6 and 7) – all are Blanks of the experiment. Each condition was performed in duplicate. Remaining white wells contained PBS or medium only to maintain humidity on the plate.

Annex 3



Annex 3. Schematic representation of a 96-well plate for optimization of cell concentration. Green and red wells represent the KBM5 and KBM5-STI cell lines, respectively, at different cell concentrations (from row 2 to 7: 2.5 x 10^5 cells/mL; 5 x 10^5 cells/mL; 8 x 10^5 cells/mL; 1.5 x 10^5 cells/mL; 3 x 10^5 cells/mL and 6 x10^5 cells/mL – all the concentrations done in duplicates). Culture medium was added to the well represented in yellow (Blank). White wells contained only PBS or medium, in order to maintain humidity on the plate.